PHOTOCHEMOTHERAPY OF VASCULAR CELLS WITH
8-METHOXYPSORALEN AND VISIBLE LIGHT:
A NOVEL APPROACH TO THE PREVENTION OF
POST-ANGIOPLASTY RESTENOSIS

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Yale University

1996

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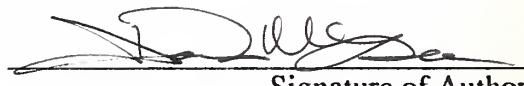
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A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

David Marshall Lee

1996

PHOTOCHEMOTHERAPY OF VASCULAR CELLS WITH 8-METHOXYPSORALEN AND VISIBLE LIGHT: A NOVEL APPROACH TO THE PREVENTION OF POST-ANGIOPLASTY RESTENOSIS

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The purpose of the present study was to evaluate the effects of photochemotherapy with 8-methoxypsoralen (8-MOP) and visible light (447 nm) on vascular cells *in vitro*. It was hypothesized that this treatment regimen could selectively inhibit smooth muscle cell proliferation and might thus represent a novel approach to the prevention of post-angioplasty restenosis.

Bovine aortic smooth muscle cells (SMC) and endothelial cells (EC) were cultured in cell wells, incubated with 1, 20, or 50 μg/mL 8-MOP, and then exposed to 12.0 J/cm^2 447 nm visible light. Control cells were wrapped with aluminum foil so as to prevent 8-MOP photoactivation. Studies were then undertaken to ascertain the effects of these treatment regimens on SMC and EC proliferation, migration, morphology, size, and photoadduct formation.

Experiments on SMC proliferation revealed no significant effect at 1 μg/mL 8-MOP, while 20 μg/mL produced a transient, reversible inhibition, and 50 μg/mL resulted in cell death. Experiments on EC proliferation also revealed no significant effect at 1 μg/mL 8-MOP; however, a transient, reversible inhibition was seen at both 20 and 50 μg/mL 8-MOP. Further experimentation revealed that photoactivated 8-MOP does not inhibit cell migration. Morphology studies demonstrated both size and phenotype changes induced by higher dosages (20 and 50 μg/mL) of 8-MOP. Cell size measurements corroborated the cell enlargement observed in the morphology studies. Followed over time, EC revealed a return to baseline sizes while SMC remained enlarged. Finally, studies on photoadduct formation revealed the formation of comparable numbers and types of photoadducts between SMC and EC.

These experiments demonstrate that while 8-MOP photoactivated with 447 nm visible light can reversibly inhibit the proliferation of both SMC and EC in a dose-dependent fashion, SMC are more sensitive to the treatments than EC. The rapidly reversible inhibition observed in EC proliferation bears clinical significance, as previous studies have demonstrated that an intact endothelium contributes to the

inhibition of SMC proliferation. As SMC proliferation constitutes a major mechanism by which restenosis after angioplasty occurs, the results of this study suggest that photochemotherapy with 8-MOP and visible light may represent a novel approach to the prevention of post-angioplasty restenosis.

Acknowledgements

I would like to begin by thanking Dr. Bauer Sumpio for his guidance and mentorship over the past few years. The time I have spent working in his lab and the things I have learned through my experiences there account for a substantial portion of my medical school training. Since the time I first set foot in his office, his model of true excellence in his own work has not stopped being a source of inspiration to me.

I also want to thank the people in the lab who have made my time working on this project a most enjoyable one—to Dr. Guangdi Li, who taught me the finer points of cell culturing, and to Drs. Wei Du and Ira Mills, backbones of the lab who were always willing to lend a helping hand.

A special thanks to Dr. Frank Gasparro for the time he invested in me and my work. His unparalleled expertise in the photobiology of psoralens and computer wizardry were invaluable in the development and completion of this project.

Thanks also to my brothers and sisters in the Yale Health Professionals Christian Fellowship. I have appreciated their prayers and encouragement throughout the years.

I cannot begin to express my gratitude to my parents and to my wife, who have persevered with me through my journey toward becoming a medical doctor. Their unconditional love and support have brought out the best in me and have been a source of immeasurable joy and strength.

Finally, I would like to give thanks and glory to God, who has granted us both the curiosity and the minds to explore the wonders of his creation. . .

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1.   Percutaneous Transluminal Coronary Angioplasty (PTCA)

The Normal Coronary Artery

Coronary artery disease remains the number one cause of morbidity and mortality in the

Western world. It results from the development of atherosclerotic lesions in the coronary

arteries, or vessels which supply the heart. The anatomy of the normal coronary artery is

no different from that of other medium-sized arteries in the human body; namely, the

vessel wall possesses three layers—an intima, media, and adventitia.
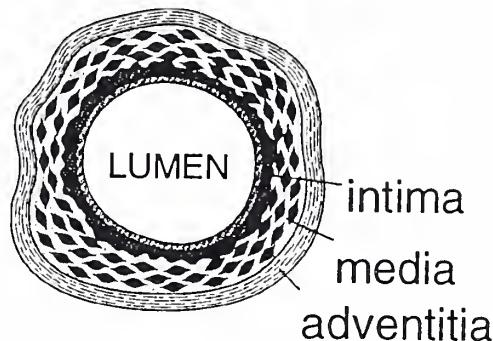
Figure 1-1. Schematic representation of the three layers of the coronary artery. Figure modified from Libby, 1992.

The intima is the layer closest to the lumen of the vessel and is primarily comprised of a monolayer of endothelial cells (EC) lining an underlying coat of connective tissue. It is separated from the media by a fenestrated membrane through which the smooth muscle cells (SMC) of the media can migrate, called the internal elastic membrane. The media, or muscular layer, of the artery forms the major structural support for the vessel and consists of several layers of SMC separated by compact, fenestrated layers of elastic tissue. A condensation of this elastic tissue at the perimeter of the media forms the external elastic membrane, which separates the media from the outermost layer of the artery, the adventitia. A poorly defined layer of connective tissue, the adventitia contains fibroblasts, elastic fibers, nerve fibers, and small nutrient vessels known as *vasa vasora*. These thin-walled vessels serve as the blood supply to the adventitial layer and to the outer portion of the media.

The Diseased Coronary Artery: Atherosclerosis

The hypothesis that an injury to the endothelium is the precipitating event in the development of atherosclerosis was first formally proposed in 1973 by Ross and Glomset (Ross and Glomset, 1973) and is still widely held today.

Commonly cited potential causes of injury include diabetes, hypertension, tobacco, radiation, and hyperlipidemia. Over the past two decades, our understanding of the pathogenesis of atherosclerosis has substantially increased and been refined by accumulating observations on the various cell types and molecules involved in atherogenesis.
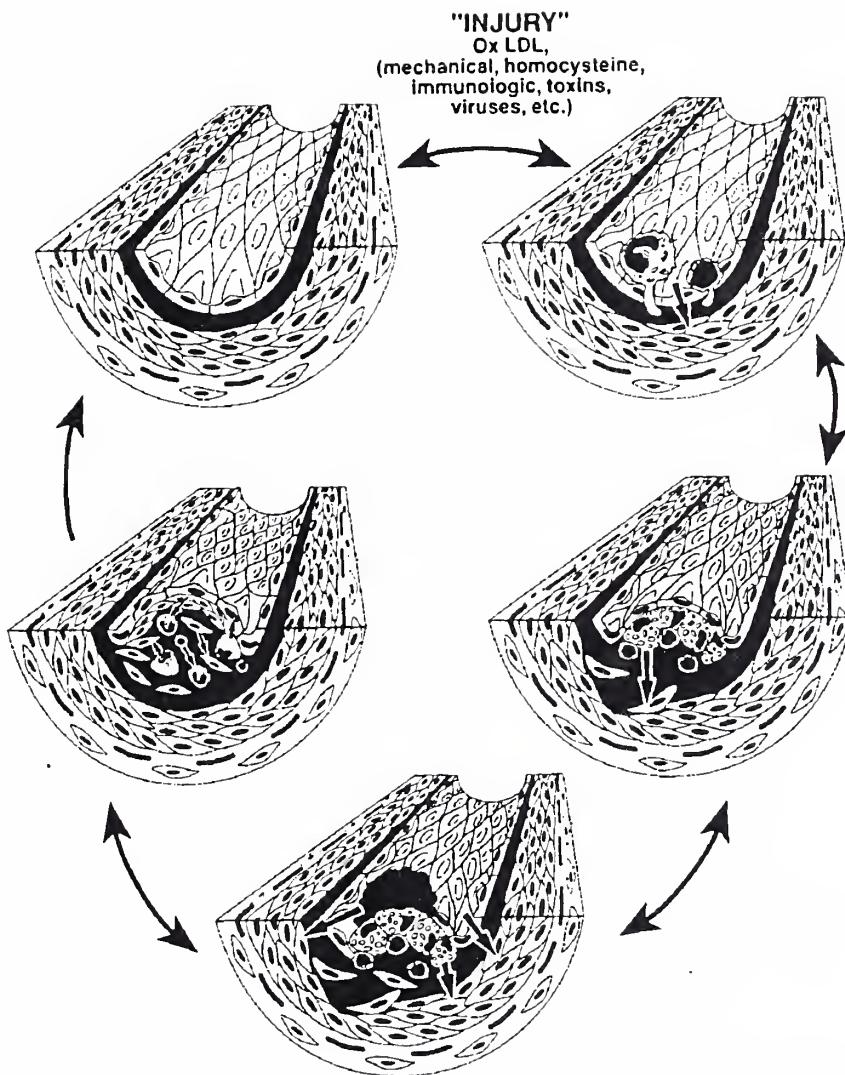
Figure 1-2. Schematic representation of the response-to-injury hypothesis of atherosclerosis. Several different sources of injury to the endothelium can lead to the formation of a fatty streak, which can ultimately progress to a fibrous plaque. Each of the stages of lesion formation is potentially reversible. Thus, lesion regression can occur if the injurious agents are removed or when protective factors intervene to reverse the inflammatory and fibroproliferative processes. Figure taken from Ross, 1993.

The earliest recognizable lesion of atherosclerosis is the "fatty streak," which consists of an aggregation of lipid-laden macrophages, or foam cells, in the intima.
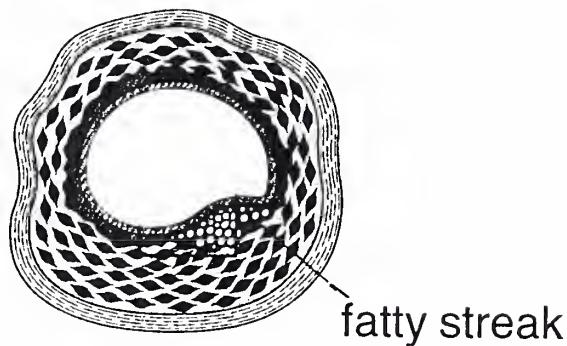
Figure 1-3. Schematic representation of a fatty streak. Figure modified from Libby, 1992.

Presumably, this lesion progresses as SMC migrate from the media into the intima and elaborate a connective tissue matrix. This results in the projection of the lesion, now termed a "fibrous plaque," into the arterial lumen. Eventually, extracellular cholesterol, calcium deposits, and necrotic cellular debris accumulate with the foam cells at the core of the lesion. Finally, the plaque is covered over by a dense fibrous cap made of connective tissue embedded with SMC (Ross, 1993; Lovqvist et al., 1993).

It is important to note that the majority of atherosclerotic lesions are eccentric rather than concentric; that is, the majority of lesions involve only a portion of the arterial circumference rather than the entire circumference (Waller, 1985).
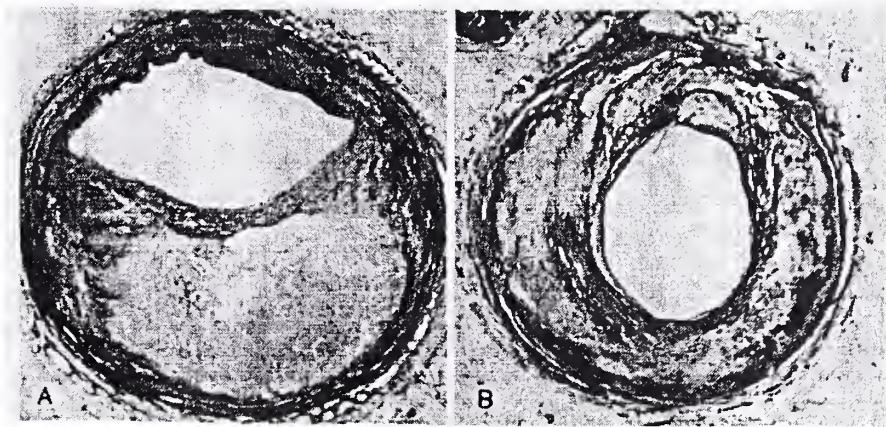
Figure 1-4. Atherosclerotic lesions in coronary arteries. A: an eccentric lesion (bottom) with disease-free segment (top). B: markedly calcified concentric plaque. Figure taken from Edwards, 1995.

An increase in size in one of these fibrous plaques causes arterial stenosis, or a narrowing of the arterial luminal cross-sectional area. This, in turn, results in an increased resistance to blood flow. When present to a sufficient degree in coronary arteries, these stenotic lesions may cause symptoms of stable angina. Fissures or ruptures in the fibrous cap, on the other hand, may result in hemorrhage into the plaque, thrombosis, and acute arterial occlusion. This latter sequence is responsible for the majority of sudden deaths caused by myocardial infarcts (Davies and Thomas, 1984).

Advances in PTCA

Non-pharmacologic therapies for symptomatic coronary artery disease include coronary artery bypass grafting (CABG) and percutaneous transluminal coronary angioplasty

(PTCA). Whereas surgical bypass is the standard of care in cases of left main coronary artery disease, triple vessel disease with compromised ventricular function, and anginal symptoms resistant to medical therapy, PTCA has been established as a significant option in the treatment of patients with coronary artery disease. Up to 50% of all patients requiring revascularization choose this considerably less invasive procedure as an alternative to bypass surgery (Ip et al., 1990).

Balloon angioplasty, the most common form of PTCA, involves the passage of a balloon along a guide wire to a site of arterial stenosis. Subsequent dilation of the balloon typically produces a stretching of plaque-free wall and fracturing or "cracking" of plaque and hence a widened lumen diameter.

First introduced in September 1977 by Andreas Gruentzig (Gruentzig et al., 1979), PTCA was initially received with skepticism. Today, it has become the treatment of choice for many patients with coronary artery disease who require revascularization (Lange et al., 1993).
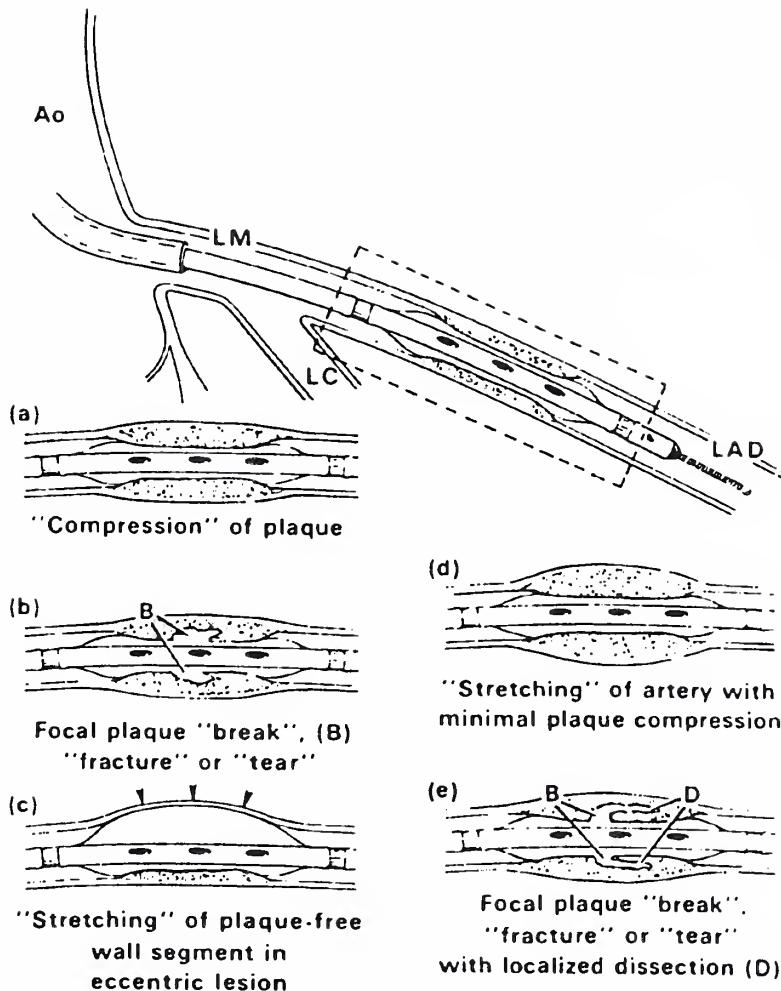
Figure 1-5. Mechanisms by which balloon angioplasty can induce revascularization. Ao = aorta; LAD = left anterior descending; LC = left circumflex; LM = left main coronary artery. Figure taken from Waller, 1985.

Formerly limited to cases involving simple single-vessel disease, PTCA has now been

applied in cases involving complex stenoses, occluded arteries, multivessel disease,

saphenous vein grafts, and acute myocardial infarction (Lange et al, 1993). Despite its

application in increasingly complex cases, improvements in technique and advances in

catheter technology have continued to improve the success rate of this procedure. With a

primary success rate of nearly 95% (Hermans et al., 1991), over 350,000 coronary

angioplasty procedures are being performed each year in the United States (Faxon and

Currier, 1995). The long-term efficacy of this minimally invasive treatment modality,

however, continues to be severely limited by the problem of post-angioplasty restenosis.

2.   Restenosis: The Achilles' Heel of PTCA

Restenosis: The Problem

Restenosis is the re-narrowing of a segment of artery formerly subjected to a

percutaneous intervention such as balloon angioplasty.
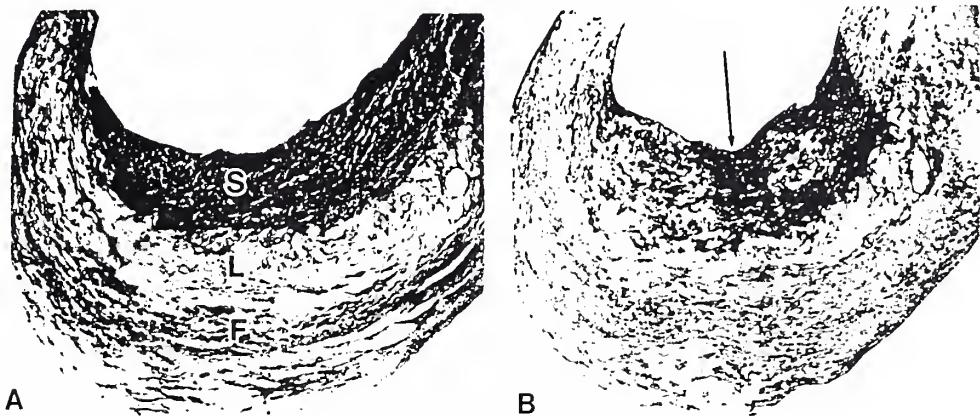
Figure 2-1. Atherectomy specimen from femoral artery of a patient who developed restenosis after balloon angioplasty. A: S = zone of SMC proliferation; L = acellular lipid core of the plaque; F = deeper fibrous region. B: arrow pointing to region of intense SMC proliferation. Figure taken from Forrester et al., 1991.

Traditionally, restenosis rates have been quoted to range anywhere from 5% to 50%. A

more contemporary view of restenosis, however, holds that it actually occurs in all

patients who undergo PTCA (Ip et al., 1991; Lovqvist et al., 1993) and that its clinical

significance is merely a matter of extent. A recent angiographic follow-up study which

revealed a near-gaussian distribution of luminal narrowing following PTCA (Rensing,

1992) supports this notion that restenosis is a continuous process rather than "all or nothing" phenomenon.
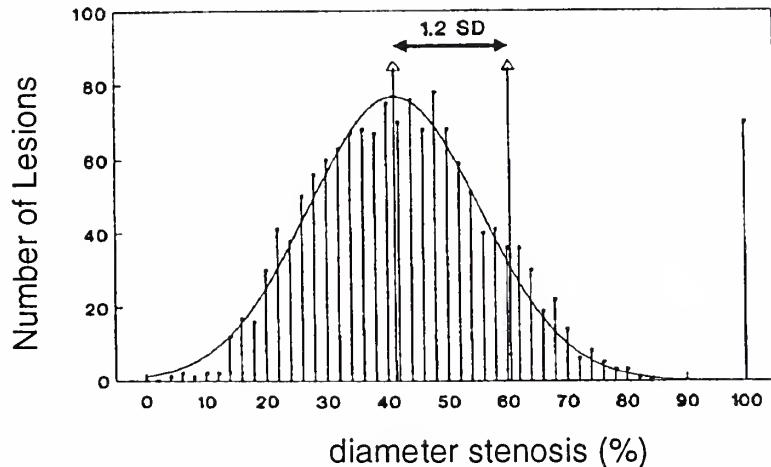
Figure 2-2. Histogram of per cent diameter stenosis seen at follow-up angiography in 1,445 lesions. The curve superimposed on the histogram represents the theoretic gaussian distribution curve. Figure modified from Rensing et al., 1992.

Restenosis: The Economic Impact

A major cause of morbidity, restenosis manifests most frequently as recurrent symptomatic angina requiring additional revascularization procedures. A treatment capable of reducing the risk of restenosis by 25% would average a calculated savings of $1400 per patient in hospital, procedural, and professional fees, while a risk reduction of 33% would save $2000 per patient (Califf, 1995). Accordingly, the magnitude of the problem of restenosis, in terms of health care costs, exceeds one billion dollars annually in the United States alone (Faxon and Currier, 1995).

Restenosis: Temporal Considerations

Restenosis can be classified as early or late depending on the histopathology of the restenosed arterial wall. Pathologic examinations of restenosis sites from patients who died after successful PTCA have revealed two types of lesion: those with only fibrous atheromatous plaques resembling the primary plaque and those with substantial intimal thickening secondary to SMC proliferation and matrix elaboration (Waller et al., 1991). The former is associated with early restenosis and the latter with late restenosis (Lovqvist et al., 1993; Lange et al., 1993).

Early Restenosis: Thrombosis, Recoil, and Vasoconstriction

Early restenosis occurs within days of PTCA. One mechanism by which it is thought to occur involves thrombus formation. The mechanical disruption of the intima caused by balloon angioplasty results in endothelial denudation of the plaque. This, in turn, promotes platelet aggregation and may precipitate the acute formation of a thrombus (Lovqvist et al., 1993).

An alternative mechanism in the pathogenesis of early restenosis involves the relaxation and vasoconstriction of stretched plaque-free portions of artery. A quantitative angiographic assessment of coronary arteries before, during, and immediately after angioplasty revealed that nearly 50% of the maximal luminal cross-sectional area achieved during PTCA is lost shortly after balloon deflation, and this presumably due to elastic recoil (Rensing et al., 1990). Additionally, the arterial vasoconstriction frequently observed at the site of coronary angioplasty (Bertrand et al., 1989) may be attributable

both to a decrease in vasodilators produced by disrupted endothelial cells and to an

increase in vasoconstrictors released by platelets (Lange et al., 1993).

Late Restenosis: Vascular Wound Healing

A complete understanding of the biologic mechanisms involved in the pathogenesis of

late restenosis has yet to be established; however, several of the mechanisms which are

involved in the process have been elucidated. Balloon angioplasty is known to inflict

substantial injury on the intima and media of the vessel wall, and it is becoming

increasingly accepted that the process of restenosis can be understood as a "wound

healing" response to such injury (Serruys et al., 1988; Forrester et al., 1991; Ip et al.,

1991; Clinton and Libby, 1992; Faxon and Currier, 1995). The ultimate result of this

wound healing response is the formation of a neointima consisting of an increased mass

of smooth muscle cells (SMC) scattered through a loose extracellular matrix. This

neointima formation is the mechanism by which late restenosis occurs.

Phase I of Vascular Wound Healing: Inflammation

It has been suggested that the process of late restenosis occurs over a series of three

phases: inflammation, granulation, and matrix formation (Forrester et al., 1991). The first

phase, known as the inflammation phase, occurs almost immediately after the injury and

continues for several days. It begins with platelet aggregation at the site of the wound

surface and thrombus formation (Wilentz et al., 1991). As the blood and soluble serum

fibronectin coagulate to form an extracellular matrix, the activated platelets release

platelet-derived growth factor (PDGF), a major stimulus of SMC migration and
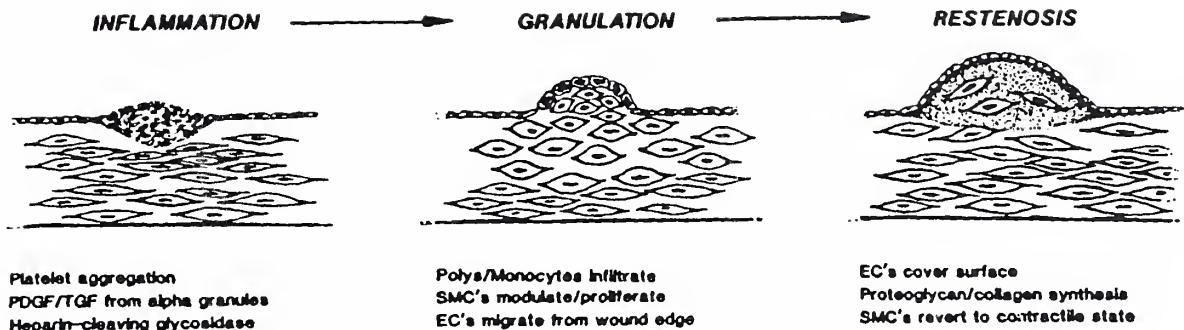
Figure 2-3. Hypothetical schema for late restenosis. PDGF = platelet-derived growth factor; TGF = transforming growth factor; Polys = polymorphonuclear cells; SMC = smooth muscle cells; EC = endothelial cells. Figure taken from Forrester et al., 1991.

proliferation, and other mitogenic factors, including epidermal growth factor and beta transforming growth factor (Ip et al., 1991). Monocytes soon collect in the area of injury and release additional growth factors (Forrester et al., 1991). Meanwhile aggregated mononuclear lymphocytes contribute cytokines such as tumor necrosis factor, interleukins, and beta transforming growth factor (Ip et al., 1991). The release of these various cytokines and growth factors results in a local milieu capable of stimulating SMC migration and proliferation and extracellular matrix production. Hence, the stage is set for the next phase of restenosis.

Phase II of Vascular Wound Healing: Granulation

The second phase of restenosis, called the granulation phase (Forrester et al., 1991), lasts approximately one week and primarily involves the migration and proliferation of SMC. Initial SMC proliferation is reported to begin as early as 24 to 48 hours following

vascular injury. Two to three days later, SMC begin to migrate to the intima, where they may continue to proliferate (Lange et al., 1993).

The stimulus for SMC migration and proliferation is multifactorial and, in addition to the presence of growth factors described above, involves endothelial disruption and direct damage to the EC and SMC. The intimal disruption associated with PTCA may promote SMC proliferation by removing the growth-inhibitory influences exerted by an intact endothelium (Ip et al., 1991). Confluent EC have been observed to secrete heparin-like glycosaminoglycans (Castellot et al., 1981), while exogenous heparin has been shown to inhibit SMC proliferation in injured arteries (Clowes and Karnowsky, 1977). Put together, these facts suggest that a functionally intact endothelium can inhibit SMC growth (Ip et al., 1990). Hence, endothelial disruption, in addition to the presence of mitogens and growth factors, may promote SMC proliferation.

Damaged EC and SMC may also contribute to the mitogenic activity of healthy SMC. EC have the ability to secrete at least two major mitogens, one of which closely resembles PDGF. Meanwhile, SMC also possess the ability to produce a PDGF-like protein. It has been reported that dying EC can produce greater than six times the amount of growth factors produced by healthy EC. Likewise, SMC isolated from injured segments of artery have been found to produce PDGF-like substances on the order of ten times that released by SMC from uninjured segments (Ip et al., 1990).

It should be noted that the SMC proliferating at the site of arterial injury are of the synthetic, rather than contractile, phenotype. Contractile-phenotype SMC predominate in

healthy adult vascular media, whereas synthetic-phenotype SMC exist in young

developing arteries. Synthetic-phenotype SMC are closely related to fibroblasts and

possess abundant synthetic organelles including free ribosomes, rough endoplasmic

reticula, and Golgi apparati (Forrester et al., 1991). They are "synthetic" in the sense that

they synthesize and secrete large quantities of extracellular matrix collagen, elastin, and

proteoglycans. These synthetic SMC are the primary cell type involved in the final phase

of the wound-healing response, the matrix formation phase.

Occurrence	SYNTHETIC PHENOTYPE	Structure	Function
embryc and young growing organism atherosclerotic lesions		euchromatic nucleus prominent ER and Golgi complex	proliferation synthesis and secretion of extracellular matrix components
normal development	↓ ↑ atherogenesis		
	CONTRACTILE PHENOTYPE		
adult organism		heterochromatic nucleus abundant actin and myosin filaments	contraction in response to chemical and mechanical stimuli

Figure 2-4. Diagrams comparing contractile- and synthetic-phenotype SMC. ER = endoplasmic reticulum. Figure taken from Thyberg et al., 1990.

Phase III of Vascular Wound Healing: Matrix Formation

The matrix formation phase of restenosis primarily involves extracellular matrix

deposition and remodeling. As the surface of the site of injury becomes covered with a

layer of cells, the SMC which have migrated to the wound area slow their proliferation

and begin to deposit large amounts of proteoglycan, most notably chondroitin sulfate and

dermatan sulfate. Neighboring EC also contribute heparin sulfate proteoglycan to the

extracellular matrix deposit. Over a course of months, these proteoglycans become

replaced by large bundles of type I collagen and elastin (Forrester et al., 1991). This

replacement of proteoglycan by collagen and elastin constitutes remodeling and signifies

the completion of the final phase of restenosis.

3.   Pharmacologic Approaches to the Prevention of Restenosis

The amount of time it takes to advance through the three phases of restenosis described in

the previous chapter is somewhat variable, but the entire process of neointima formation

is reported to be largely complete by 90 to 120 days (Forrester et al., 1991).
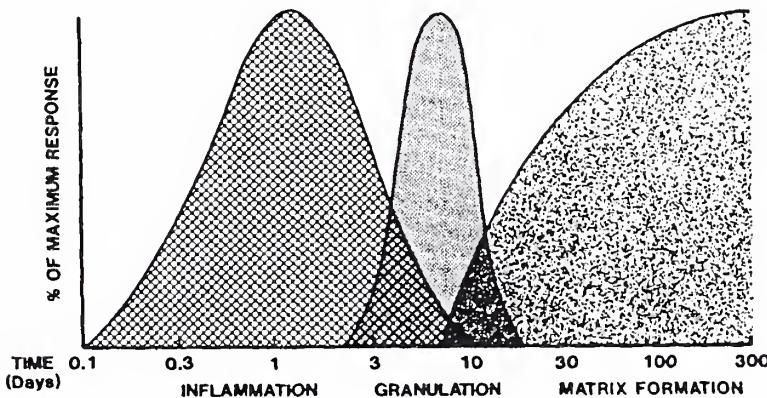
Figure 3-1. Time course for the three phases of wound healing. Figure modified from Forrester et al., 1991.

The fact that this process occurs over a multiplicity of steps suggests that the problem of

restenosis can potentially be addressed at a number of levels. Accordingly, a variety of

drugs aimed at reducing platelet adhesion, thrombosis, fibrin formation, growth factor

expression, and SMC proliferation have been suggested (Ip et al., 1990; Hermans et al.,

1991).

Multiple classes of drug have been studied in experiments aimed at reducing or

preventing post-angioplasty restenosis. These include antiplatelet agents, antithrombotic

agents, lipid-lowering agents, antispamodic agents, antineoplastic agents, and

antiproliferative agents. It should be noted that while many approaches have successfully

inhibited restenosis in rat or rabbit models, none have proven effective in pig or primate

models, and none have been successful at preventing restenosis in human clinical trials

(Wilcox et al., 1993).

Pharmacologic Approaches to Post-angioplasty Restenosis

aspirin	dipyridamole
ticlopidine	prostacyclin
thromboxane A2 inhibitors	monoclonal antibodies to GP IIb/IIIa
heparin	low molecular weight heparin
warfarin	hirudin
cholesterol synthesis inhibitors	fish oils
nifedipine	diltiazem
vincristine	actinomycin D
methotrexate colchicine	glucocorticoids
cilazapril	angiopeptin
trapidil	

Table 3-1. Summary of some of the different agents used in experiments designed to reduce or prevent restenosis after angioplasty.

Antiplatelet Agents

The antiplatelet agents studied have included aspirin, dipyridamole, ticlopidine,

prostacyclin, thromboxane A2 inhibitors, and monoclonal antibodies to the platelet

receptor GP IIb/IIIa. The theoretical basis for their use in strategies designed to prevent

post-angioplasty restenosis is founded on the premise that inhibition of platelet deposition

may reduce SMC exposure to PDGF and other growth factors which can stimulate

neointima formation. One study examining the role of platelets in post-angioplasty

restenosis revealed that thrombocytopenic rabbits experienced a marked reduction in

intimal thickening following balloon-induced arterial injury (Friedman et al., 1977).

Aspirin, dipyridamole, and ticlopidine. Aspirin inhibits platelet aggregation by

irreversibly acetylating the enzyme cyclooxygenase, thereby suppressing the synthesis of

proaggregatory thromboxane A2. Dypyridamole acts by increasing intracellular levels of

cyclic AMP via the inhibition of cyclic nucleotide phosphodiesterase, which in turn may

potentiate prostacyclin's ability to antagonize platelet adhesion to thrombogenic surfaces.

While the combination of aspirin and dipyridamole has been shown to decrease the

incidence of acute complications both during and after PTCA, it has proven ineffective in

preventing restenosis (Schwartz et al., 1988; Chesebro et al., 1989). Ticlopidine, which

acts by altering the platelet membrane such that ADP-induced exposure of the fibrinogen

binding site on platelet glycoprotein IIb/IIIa is prevented, is another platelet inhibitor.

Studies using this agent have also failed to demonstrate beneficial effects on restenosis

(White et al., 1987).

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Prostacyclin and ciprostene. Prostacyclin is a naturally occurring arachidonic acid

metabolite which possesses both platelet inhibitory and vasodilatory properties.

Administration of prostacyclin shortly before PTCA and continued for 48 hours after

failed to reduce the incidence of restenosis (Knudtson et al., 1990). Likewise, short-term

administration of ciprostene, a prostacyclin analogue, failed to affect the incidence of

angiographically-determined restenosis, although some clinical benefit was noted

(Raizner, 1988).

Thromboxane A2 inhibitors. In addition to its proaggregatory effects on platelets,

thromboxane A2 has been shown to have a mitogenic effect on vascular SMC (Hanasaki

et al., 1990). Thromboxane A2 inhibitors work by blocking the formation of thromboxane

and redirecting the metabolism of prostaglandin endoperoxides toward prostacyclin

synthesis. A small, promising study suggested that thromboxane A2 synthetase inhibitors

might prove beneficial in preventing restenosis (Yabe et al., 1989). However, a

subsequent randomized, double-blind, placebo-controlled trial involving blockade of the

thromboxane A2-receptor revealed no reduction in incidence of restenosis (Serruys et al.,

1991).

Antibodies to GP IIb/IIIa. Glycoprotein IIb/IIIa is a platelet membrane receptor which

plays an important role in the adhesion of platelets to the vessel wall (Ip et al., 1990). It is

also involved in the binding of fibrinogen to platelets (Lovqvist et al., 1993). While the

administration of monoclonal antibodies to GP IIb/IIIa has been found to prevent acute

thrombosis in the canine model (Bates et al., 1988), its effectiveness in preventing post-

angioplasty restenosis in humans has not been demonstrated and is currently being evaluated in ongoing clinical trials (Faxon and Currier, 1995).

Antithrombotic Agents

The antithrombotic agents studied have included heparin, low molecular weight heparin, warfarin, and hirudin. The theoretical basis for their use in strategies designed to prevent post-angioplasty restenosis involves the role that thrombus formation plays in both early and late restenosis. As described in the previous chapter, acute thrombosis represents a mechanism by which early restenosis may occur. Moreover, during thrombosis, thrombin may become incorporated into both the arterial extracellular matrix and the thrombus itself and can be gradually released during fibrinolysis. As thrombin has been demonstrated to have mitogenic activity for SMC (Ip et al., 1991), it may thus play an important role in the long-term stimulation of SMC proliferation.

Heparin. As mentioned previously, heparin has been found to inhibit SMC proliferation *in vivo* (Clowes and Karnowsky, 1977). While standardly administered to patients during and in the hours following PTCA to prevent acute coronary thrombosis (Lange et al., 1993), heparin given for 18 to 24 hours has failed to demonstrate a reduction in incidence of restenosis (Ellis et al., 1989).

Low molecular weight heparin. Low molecular weight heparin was found to reduce restenosis after angioplasty in the rabbit model (Pow et al., 1989). However, one-month treatments with subcutaneous low molecular weight heparin in a double-blind randomized trial have proven ineffective at preventing restenosis (Faxon et al., 1992).

Warfarin. Warfarin is a vitamin K antagonist which inhibits coagulation and thrombin formation. Several small clinical trials evaluating the effects of warfarin given after angioplasty have failed to demonstrate a decrease in restenosis (Franklin, 1993).

Hirudin. Hirudin is a potent antithrombotic agent which has proven to be more effective than heparin in preventing thrombosis in animal models (Lovqvist et al., 1993). Its effectiveness in preventing post-angioplasty restenosis has yet to be demonstrated (Faxon and Currier, 1995).

Lipid-lowering Agents

The lipid-lowering agents studied have included cholesterol synthesis inhibitors and fish oils. The theoretical advantage of a reduction in lipids in the prevention of post-angioplasty restenosis is based on the paradigm of accelerated atherosclerosis as a model for restenosis (Ip et al., 1990).

Lovastatin. Lovastatin, a cholesterol synthesis inhibitor with antiprloiferative properties (Gellman et al., 1991), had previously shown promise in its ability to reduce restenosis in a randomized clinical trial (Sahni et al., 1989). A recently-completed large double-blind controlled trial, however, casts doubt on its effectiveness at preventing post-angioplasty restenosis (Weintraub et al., 1992).

Fish oils. Fish oils, rich in omega-3 fatty acids, are known to decrease LDL cholesterol while simultaneously increasing HDL cholesterol. It has been suggested that fish oils may reduce SMC proliferation by inhibiting EC production of a PDGF-like protein (Fox and

DiCorleto, 1988). Several randomized clinical trials designed to evaluate the effects of fish oils on restenosis have been performed and offer conflicting results. While nearly half of the clinical trials to date involving fish oils have demonstrated some beneficial reduction in restenosis (Franklin et al., 1993), a recently-completed randomized controlled clinical study involving large doses of omega-3 fatty acids with a minimum of two weeks pretreatment revealed that fish oils were not effective at inhibiting restenosis (Faxon and Currier, 1995).

Antispasmodic Agents

The antispasmodic agents studied have included the calcium channel blockers nifedipine and diltiazem. The theoretical basis for their application in strategies designed to prevent post-angioplasty restenosis involves the contribution of coronary vasoconstriction and vasospasm to the process of early restenosis.

Nifedipine and diltiazem. Despite some promising results in animal models, clinical trials in humans have shown that neither nifedipine nor diltiazem demonstrates a beneficial effect on restenosis (Whitworth et al., 1986; O'Keefe et al., 1991).

Antineoplastic Agents

The antineoplastic agents studied have included vincristine, actinomycin D, and methotrexate. The rationale for their use in strategies designed to prevent post-angioplasty restenosis is based on the ostensible ability of these agents to selectively injure dividing SMC while leaving non-proliferating cells undamaged (Lovqvist et al., 1993).

Vincristine, actinomycin D, and methotrexate. While successful in preventing SMC proliferation after endothelial injury in animals (Barath et al., 1989), the potential clinical toxicity of these drugs prohibits their realistic application in the human model.

Antiproliferative Agents

The antiproliferative agents studied have included colchicine, glucocorticoids, cilazapril, angiopeptin, and trapidil. The theoretical basis for their use in strategies designed to prevent post-angioplasty restenosis is derived from the principal role that SMC proliferation plays in the process of late restenosis.

Colchicine. Colchicine interferes with proper microtubule function by binding tubulin, the subunit protein of microtubules. Consequently, it is a potent inhibitor of cell division. While successful at preventing restenosis after balloon injury in various animal models (Bilazarian et al., 1989), a recent randomized, placebo-controlled trial has shown that colchicine is ineffective at preventing restenosis in humans (O'Keefe et al., 1992).

Corticosteroids. Steroids have well-characterized antiinflammatory and antiproliferative properties. While hydrocortisone has demonstrated a synergistic inhibition of SMC proliferation when combined with low molecular weight heparin (Gordon et al., 1987), a multicenter controlled trial has revealed that corticosteroids yield no beneficial effect on restenosis (Pepine et al., 1990).

Cilazapril. In addition to being a vasoconstrictor, angiotensin II is able to stimulate SMC proliferation and matrix formation (Lange et al., 1993). Cilazapril, an angiotensin

converting enzyme (ACE) inhibitor, has been shown to reduce restenosis in experimental angioplasty (Bilazarian et al., 1991). However, two large multicenter trials evaluating the long-term effects of cilazapril on restenosis have shown no difference between treated and control patients (MERCATOR, 1992; Faxon et al., 1992).

Somatostatin and angiopeptin. Somatostatin is a known potent inhibitor of growth hormone secretion. Angiopeptin, a somatostatin analogue, has been shown to inhibit myointimal proliferation in animal models following endothelial injury (Lundergan et al., 1991). Multicenter trials in both Europe and the United States, however, have failed to demonstrate any beneficial impact of angiopeptin on post-angioplasty restenosis (Faxon and Currier, 1995).

Trapidil. Trapidil (triazolopyrimidine) possesses antiproliferative effects by virtue of its ability to antagonize PDGF. Its success in preventing post-angioplasty restenosis in atherosclerotic rabbits (Liu et al., 1990) has prompted clinical trials. While two small studies have shown trapidil to be effective at preventing restenosis (Okamoto et al., 1992; STARC, 1993), further investigation is warranted.

4.   Photochemotherapy with 8-MOP and visible light

Photochemotherapy

Photochemotherapy is a therapeutic modality that makes use of drugs which can be

pharmacologically activated by light. Upon irradiation, these drugs which possess low

intrinsic activity are transformed into highly reactive compounds. Photochemotherapy has

been used safely and effectively in the treatment of a variety of hyperproliferative skin

disorders for centuries. More recently, studies have been performed to assess the potential

efficacy of a variety of photochemotherapeutic agents in the control of SMC proliferation.

Chloroaluminum-sulfonated phthalocyanine. Chloroaluminum-sulfonated

phthalocyanine, a photosensitizer currently under evaluation for the treatment of

malignant lesions, has been shown to effectively reduce intimal hyperplasia in a rat model

(Ortu et al., 1992). While this treatment regimen demonstrates promise for potential

application in the prevention of post-angioplasty restenosis, its effectiveness in the human

model has yet to be investigated.

Hematoporphyrin compounds. Both *in vivo* and *in vitro* studies on the effects of

hematoporphyrin compounds on SMC proliferation have also suggested their potential

use in therapies designed to inhibit neointima formation (Litvack et al., 1985; Dartsch et

al., 1990). These compounds, however, are associated with a cutaneous phototoxicity for

up to several weeks after administration. This has, in general, limited their clinical application to experimental treatments involving certain malignant cancers (March et al., 1993).

Photofrin. Photofrin is yet another photosensitizer which has been shown to successfully reduce intimal hyperplasia in animals. Photoactivated with 630 nm light, Photofrin was shown to decrease intimal thickening in a rabbit model by approximately 50% (Eton et al., 1992). The toxicity of Photofrin in humans, however, has yet to be resolved. Moreover, a recent study demonstrating that photoactivated Photofrin can induce microtubule depolymerization in human EC (Sporn and Foster, 1992) calls its suitability for human application into question. As damaged EC contribute to the process of restenosis by stimulating SMC proliferation (see "Phase II of Vascular Wound Healing: Granulation," Chapter 2), a more ideal treatment regimen might entail the use of a drug which could selectively inhibit SMC proliferation without causing damage to EC.

8-Methoxypsoralen (8-MOP)

Photochemotherapy with 8-methoxypsoralen (8-MOP) dates as far back as the 15th century B.C. Ancient manuscripts document the use of this compound, extracted from psoralen-containing plants, in the treatment of vitiligo. Photoactivation during this time was achieved via exposure to natural sunlight (Roelandts, 1991).

Today, photochemotherapy with 8-MOP has been approved by the Food and Drug Administration (FDA) for the treatment of a variety of skin diseases. Treatment typically

consists of oral or intravenous administration of 8-MOP followed by photoactivation with high-intensity UVA bulbs in affected areas of skin.

Given its excretion rate of approximately 90% within the first 12 hours of administration (Gupta and Anderson, 1987), 8-MOP exhibits minimal long-term phototoxicity. Its established long-term safety and therapeutic efficacy have helped it to become the foremost photosensitizer employed in all of photomedicine (Gasparro, 1990). While photochemotherapy with 8-MOP is most commonly used in the treatments of psoriasis and vitiligo, its clinical application has spanned more than 40 other diseases (Roelandts, 1991).

Clinical applications of photochemotherapy with 8-MOP

acne vulgaris	ichthyosis
alopecia areata	impetigo herpetiformis
atopic dermatitis	lichen planus
basal-cell and squamous cell carcinoma	lymphomatoid papulosis
chronic actinic dermatitis	mycosis fungoides
chronic hyperkeratotic dermatitis	nodular prurigo
chronic urticaria	palmoplantar pustulosis
contact dermatitis	papuloerythroderma
Darier's disease (keratosis follicularis)	parapsoriasis
disseminated superficial actinic porokeratosis	pityriasis alba
dyshidrotic eczema	pityriasis lichenoides
epidermodysplasia verruciformis	pityriasis rubra pilaris
erythema elevatum et diutinum	polymorphous light eruption
erythrodermia congenitalis progressiva symmetrica	psoriasis
erythropoietic protoporphyria	purpura
follicular mucinosis	scleromyxedema
graft-versus-host-reaction	seborrheic dermatitis
granuloma annulare	solar urticaria
granuloma faciale	acantholytic dermatosis
Hailey-Hailey disease	urticaria pigmentosa
herpes	vitiligo
hydroa vacciniforme	warts
hyperkeratosis lenticularis persistans	

Table 4-1. Summary of some of the indications for which photochemotherapy with 8-methoxypsoralen has been applied. Table modified from Roelandts, 1991.

8-MOP is a planar tricyclic furocoumarin. Its 5-member furan ring and 6-member pyrone ring are connected linearly by a benzene ring.
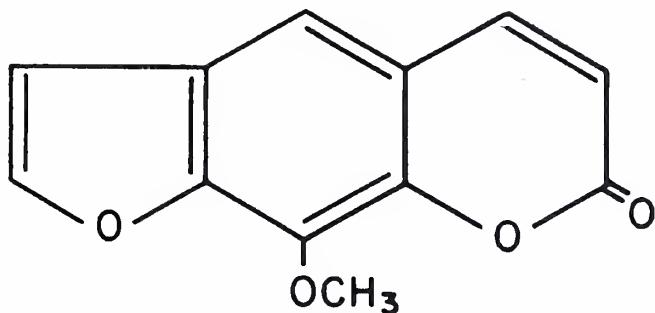

Figure 4-1. Diagram of 8-methoxypsoralen. Figure taken from Gasparro, 1994.

When exposed to cells in darkness, 8-MOP molecules can easily diffuse into the nuclei and intercalate between DNA base pairs. While inert in the dark, 8-MOP forms photoadducts with DNA when exposed to light. These photoadducts represent the key to 8-MOP's effectiveness in anti-proliferative photochemotherapies, as they presumably cause an arrest in DNA synthesis (Roelandts, 1991) and hence cell replication.
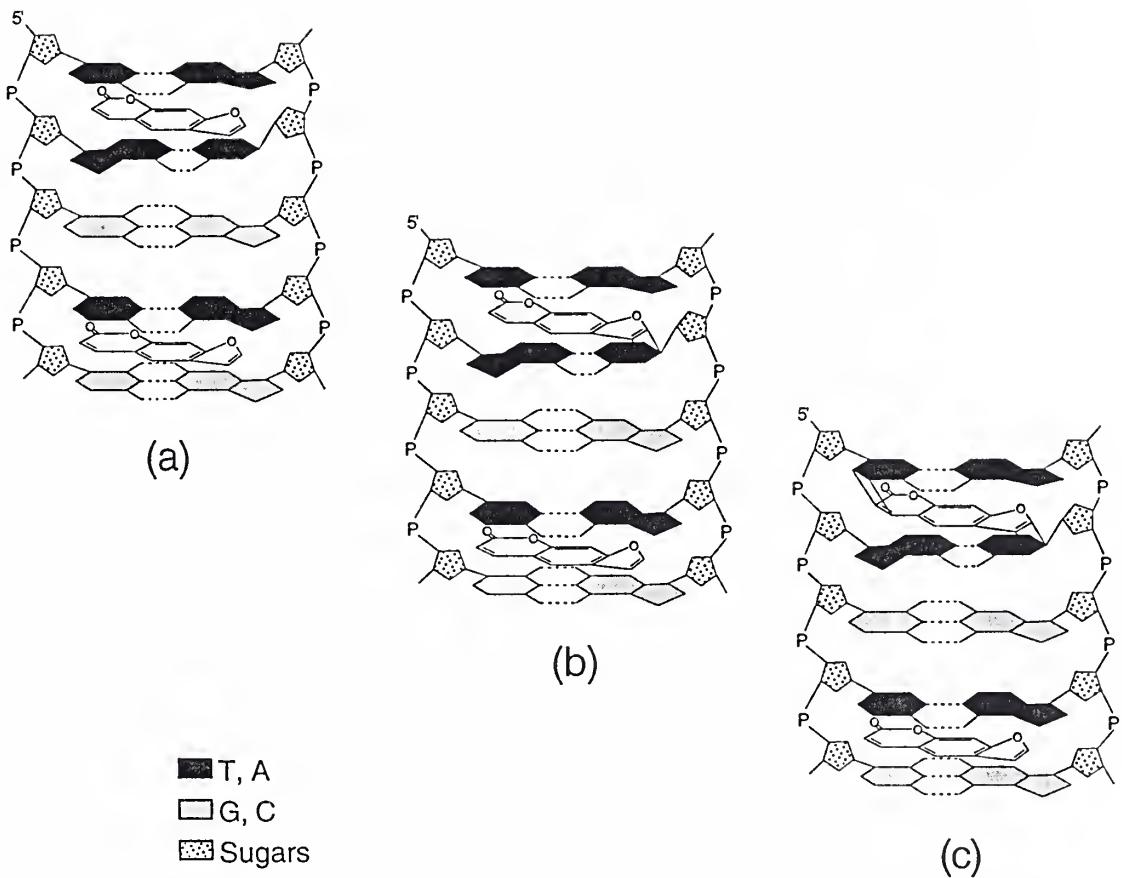
Figure 4-2. Schematic demonstrating photoadduct formation by 8-MOP. a: Two 8-MOP molecules intercalated between DNA base pairs. As 8-MOP forms photoadducts primarily with thymine, the upper molecule is positioned to form a crosslink, while the lower molecule is positioned to form a 3, 4-monoadduct. b: the upper molecule forms a 4', 5'-monoadduct with the DNA. c: additional formation of a 3, 4-monoadduct converts the former 4', 5'-monoadduct into a crosslink. Not all 8-MOP molecules form photoadducts with the DNA, as seen in the lower molecule. Figures taken from Gasparro, 1994.

Three distinct types of photoadduct form between 8-MOP and DNA upon light-

activation. These include two types of monoadduct (a 4', 5'-monoadduct and 3, 4-

monoadduct) and a crosslink.
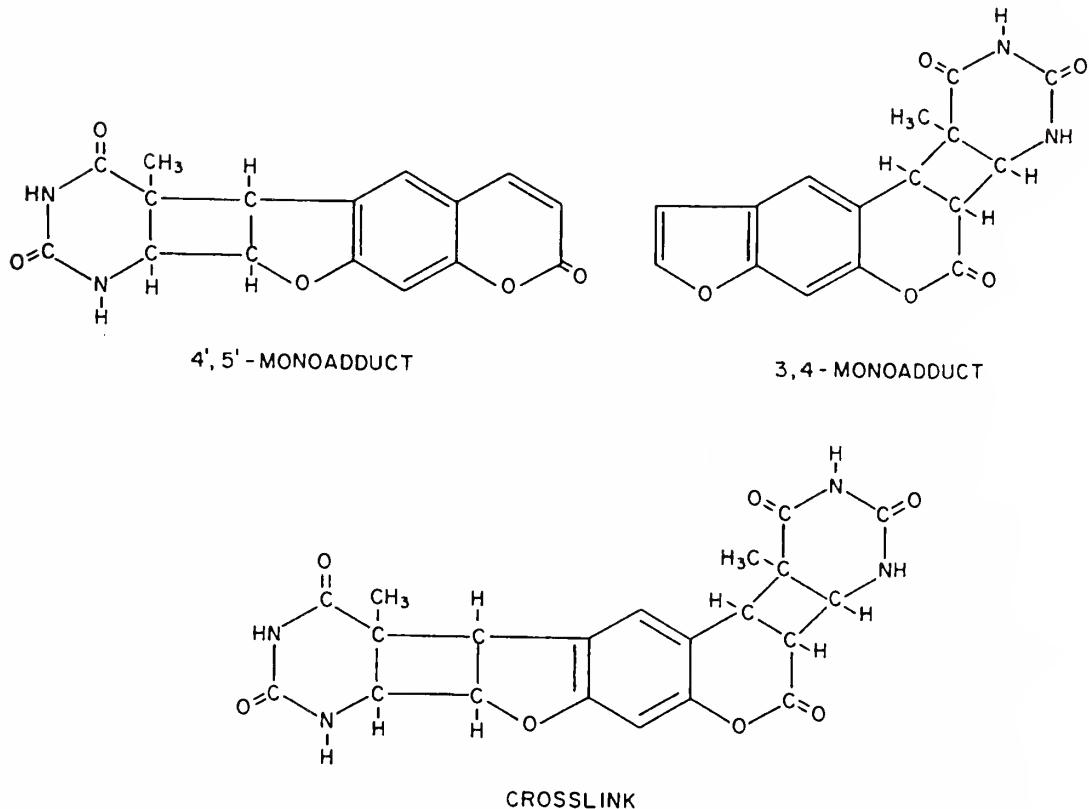
Figure 4-3. Diagrams of the 4', 5'-monoadduct; 3, 4-monoadduct; and crosslink. Figure taken from Gasparro, 1994.

All three photoadducts are the product of 2+2 photocycloadditions to pyrimidine bases,

primarily thymine (Gasparro et al., 1990).

The absorption spectrum of 8-MOP has been measured over the range of 200-500 nm

with a peak absorbance found at the 200-300 nm wavelengths (Gasparro et al., 1993).
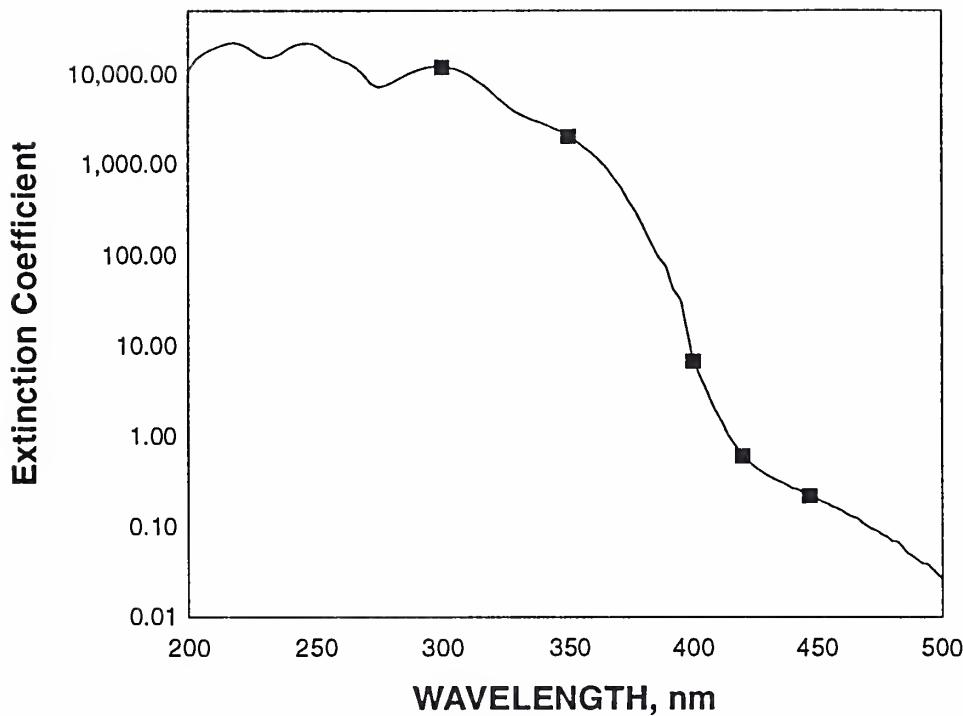
Figure 4-4. Absorption spectrum for 8-MOP. Note the extinction coefficients corresponding to the wavelengths with the filled squares are: 300 nm, 11800 cm^{-1} M^{-1}; 355 nm, 2016 cm^{-1} M^{-1}; 400 nm, 6.8 cm^{-1} M^{-1}; 419 nm, 0.60 cm^{-1} M^{-1}; and 447 nm, 0.22 cm^{-1} M^{-1}. Figure taken from Gasparro, 1994.

While 8-MOP has traditionally been activated with long-wavelength ultraviolet radiation, recent studies have shown that photoactivation with visible light results in a significant reduction in crosslink formation. The percentage of crosslinks formed by 8-MOP photoactivated at 419 nm is less than one fourth that formed when activated with ultraviolet radiation (UVA, 320-400 nm), while the percentage of crosslinks formed by 8-

MOP photoactivated at 447 nm is almost one fiftieth that formed when activated with

UVA.

Adduct Formation and Distribution in SMC

Wavelength (dose) nm (J/cm^2)	[8-MOP] $\mu g/mL$	Total Adducts per mbp	4',5'-MA	3,4-MA	XL
UVA (2)	1.0	88.0	41%	12%	47%
419 nm (7)	1.0	9.0	84%	13%	3%
419 nm (12)	1.0	13.5	78%	12%	11%
447 nm (12)	20.0	22.4	90%	9%	1%

Table 4-1. Adduct formation and distribution in SMC. MA = monoadducts; XL = crosslinks. Table modified from Sumpio et al., 1994.

It has been postulated that this reduction in crosslink formation may serve to reduce the

drug's mutagenicity and hence enhance its therapeutic efficacy (Gasparro et al., 1993;

Sumpio et al., 1994).

5.   Statement of Purpose

The purpose of the present study was to address the problem of post-angioplasty restenosis by evaluating the effects of 8-MOP photoactivated with 447 nm visible light on vascular cells (SMC and EC) *in vitro*.

The photosensitizer 8-MOP was chosen for its established long-term safety. As described in the previous chapter, this FDA-approved drug is commonly used and well-tolerated in therapies for a variety of dermatologic diseases. Its administration is associated with minimal long-term phototoxicity secondary to a rapid clearance rate.

Photoactivation with visible light rather than ultraviolet light was favored for the significant reduction in crosslink formation associated with visible-light photoactivation (Gasparro et al., 1993; Sumpio et al., 1994). Additionally, visible light was chosen as it offers both a deeper tissue penetration and superior compatibility with fiber-optic applications than ultraviolet light.

The 447 nm wavelength used in the experiments was chosen primarily for the immediate availability of the 447 nm laser. This laser is presently available on the market, as it is being manufactured for application in other clinical scenarios.

It is hypothesized that photochemotherapy with 8-MOP and visible light can result in the selective inhibition of SMC proliferation and may thus represent a novel approach to the prevention of post-angioplasty restenosis.

6.   Experimental Methods[a]

Cell Culture

Bovine SMC and EC were obtained from calf thoracic aortas by means of a previously

described explant method (Sumpio and Banes, 1988). Thoracic aortas were removed

aseptically from calves and transferred on ice to a tissue culture facility. Under a laminar

flow hood, the peri-aortic fat was carefully trimmed and discarded. A longitudinal

incision was then made along the length of the aorta and the lumen exposed. After being

rinsed five to six times with Phosphate Buffered Saline (PBS), the EC were carefully

removed with a blade and placed in a culture dish containing growth media consisting of

Dulbecco's Modified Eagle's Medium F-12 (DMEM F-12) supplemented with antibiotics

(300 U/mL penicillin, 300 U/mL streptomycin), deoxycytidine/deoxythymidine, and 10%

(v/v) heat-inactivated fetal bovine serum. Following removal of the EC, the exposed

medial layer of aorta was rinsed thoroughly with PBS and 2 mm^2 biopsies obtained and

placed in a culture dish containing growth media consisting of Dulbecco's Modified

Eagle's Medium (DMEM) supplemented with antibiotics (300 U/mL penicillin, 300

U/mL streptomycin), 0.2 M L-glutamine, and 10% (v/v) heat-inactivated fetal bovine

serum.

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[a] All procedures described in this methods chapter, barring those described in the Size and Photoadduct Formation section, were performed by the author.

Both cell types were then incubated at 37° C and 5% CO_2 until explants could be detected. The presence of EC or SMC was confirmed by direct visualization via light microscopy. SMC were identified by their typical hill-and-valley formation at confluency and hence could be distinguished from fibroblasts, which exhibit a whorl-like appearance.

Both cell types were subcultured with 0.01% trypsin/ethylene diamino tetraacetic acid (EDTA) and cultured on 75 cm^2 tissue culture flasks, maintained in their respective growth media as described above, and grown at 37° C and 5% CO_2. Cells used in the experiments were obtained from subsequent passages subcultured with 0.01% trypsin/EDTA. All experiments were performed using cells of passage seven or less.

Chemicals

A 1 mg/mL stock solution of 8-MOP (Sigma Chemical Co., St. Louis, MO) was prepared in absolute ethanol. The different concentrations of 8-MOP used in the experiments were obtained by appropriate dilution of this stock and subsequently verified by spectroscopy or high-performance liquid chromatography (HPLC) analysis.

Experimental Protocol

Bovine aortic SMC or EC were detached and collected from the tissue culture flasks by trypsinization with 0.01% trypsin/EDTA. They were then seeded in polystyrene 12-well plates at a density of 5,000 to 10,000/cm^2 and placed in an incubator at 37° C and 5% CO_2. Following a 24-hour attachment period, the cells were rinsed once with Phosphate Buffered Saline (PBS) and then incubated with 1, 20, or 50 µg/mL 8-MOP for 30 minutes

in the dark. The plates containing control cells were wrapped with aluminum foil prior to incubation so as to prevent ambient light exposure.

All plates, including the control plates still wrapped in foil, were then exposed to 12.0 J/cm^2 visible light (447 nm) in a photochemical reactor (Southern New England Ultraviolet Co., Branford, CT) equipped with lamps spaced evenly over an arc at 22.5^o intervals. The light doses being administered were determined using a calibrated silicon diode UV250BQ (EG&G, Montgomeryville, PA). Following irradiation, the 8-MOP media was aspirated and replaced with fresh growth media, and the plates were returned to the incubator. Growth media was aspirated and replaced with fresh media every 48 hours.

Cell Counts

Cell counts were performed on days 0, 1, 3, 5, 7, 10, and 14 following the initiation of each experiment. Growth media from each cell well was collected and later analyzed for the presence of LDH to ascertain cell death. Meanwhile, SMC or EC from each cell well were rinsed once with PBS, detached by treatment with 1% trypsin/EDTA, and collected in aliquots. The number of cells in each aliquot was then determined using a Coulter Counter (Model ZM, Hiahleah, FL).

Cell Migration

SMC or EC were collected and seeded in Nunc 2-well chamber slides at near confluent densities (approximately $70,000/cm^2$). They were then incubated for an attachment period

of 48 hours. A second set of cells was incubated for an additional 2 hours in 10 μg/mL mitomycin C. The purpose of the mitomycin was to inhibit the potentially confounding effect of cell proliferation on cell migration.

All cells were then rinsed with PBS, treated with 8-MOP, and exposed to light in the same manner as described previously. Cells treated with 0 μg/mL 8-MOP and exposed to visible light served as the control cells for these experiments. Before the 8-MOP media was aspirated and replaced with fresh growth media, a sterile wound was created across the middle of the well using a sterile blade, and the cells on one side of the wound were completely removed. On the specified days, the appropriate cells were rinsed with PBS, fixed with 10% formalin, and stored in PBS. At a later time, they were stained with 2.5% (v/v) crystal violet for 1 minute, rinsed twice with PBS, and then examined and photographed under a light microscope.

Cell Morphology

SMC were collected and seeded in Nunc 2-well chamber slides at a density of approximately 10,000 to 20,000/cm^2. They were then incubated for an attachment period of 24 hours. Afterwards, the cells were rinsed with PBS, treated with 8-MOP, exposed to light, incubated, and maintained in the same manner as described in the Experimental Protocol section. On the specified days, the appropriate cells were rinsed with PBS, fixed with 10% formalin, and stored in PBS. At a later time, they were stained with 2.5% (v/v) crystal violet for 1 minute, rinsed twice with PBS, and then examined and photographed under a light microscope.

Cell Size[b]

SMC and EC were collected and seeded in polystyrene 12-well plates at a density of 5,000 to 10,000/cm^2 and allowed to attach over a 24 hour period. Treated cells were incubated with 20 µg/mL 8-MOP for 30 minutes in the dark and then exposed to 12.0 J/cm^2 447 nm visible light. As in other experiments, cells treated with 8-MOP but not exposed to light served as control cells. Cells neither treated with 8-MOP nor exposed to light and cells not treated with 8-MOP but exposed to light served as additional controls for this set of experiments.

Cell size was measured with a Coulter Counter equipped with a Channelyzer (Coulter, Hiahleah, FL) on days 3, 5, 7, 10, and 14 following the initiation of each experiment. SMC or EC from the cell wells were rinsed once with PBS, detached by treatment with 1% trypsin/EDTA, and then collected in 500 µL aliquots. The sizes of 10^4 cells per aliquot were measured and recorded.

Photoadduct Formation[b]

SMC and EC were collected and seeded in polystyrene 12-well plates at a density of 5,000 to 10,000/cm^2. The cells were then incubated with 20 µg/mL of 8-MOP including trace amounts of [^{3}H]8-MOP (specific activity = 83 Ci/mmol; Amersham International, Arlington Heights, IL) for 30 minutes in the dark and irradiated with 32.0 J/cm^2 447 nm visible light. Afterwards, the cells were trypsinized and suspended in 500 µL of 50

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[b] Studies performed with the help of Xiu-Jie Wang and Francis Gasparro.

mmol/L TRIS/150 mmol/L NaCl/100 mmol/L EDTA (pH 8), and the DNA was isolated as described previously (Bevilacqua et al., 1991; Olack et al., 1993). DNA concentration was determined by UV spectroscopy (LKB UltroSpec II), and the molar concentration in terms of nucleotide units (base pairs) was computed from the absorbance at 260 and 280 nm.

The DNA was hydrolyzed enzymatically with 1 Unit/mL P_1 nuclease and 0.1 Unit/mL DNase I in water and then applied to an ODS reversed phase column (Regis Rexchrome, 4.6x250 mm; 5 micron, Morton Grove IL) for analysis. The [^{3}H] 8-MOP photoadducts (4',5' monoadducts; 3,4 monoadducts; and cross-links) were detected by scintillation analysis of HPLC fractions collected during the analysis of enzymatically hydrolyzed DNA isolated from cells.

Statistical Analysis

Statistical analyses (ANOVA and Student's t Test) were performed using Systat for Windows (Evanston, IL) and Sigmaplot (Jandel, San Rafael, CA).

7.   Results

SMC Proliferation

The effects of 12.0 J/cm^2 447 nm visible light-activated 8-MOP on SMC proliferation are shown in the following representative growth curves (Figures 7-1, 7-2, and 7-3). The lowest dose of 8-MOP (1 µg/mL) had no effect on SMC proliferation (Figure 7-1). The intermediate dose of 8-MOP (20 µg/mL) yielded transient and reversible inhibition of SMC proliferation, as evidenced by the recovery in the growth curve (Figure 7-2). The highest dose of 8-MOP (50 µg/mL) was cytotoxic to the SMC (Figure 7-3). The growth of SMC treated with drug or light alone at each dose was not significantly different from control, untreated SMC (data not shown). The results of triplicate experiments per drug dose were combined and expressed as percent recovery for days 3, 5, 7, and 10 (Figure 7-4), where percent recovery represents the number of cells per aliquot divided by the number of cells in the corresponding control aliquot. Differences in percent recovery between cells treated with 1 µg/mL and the two higher doses were statistically significant (p<0.05).
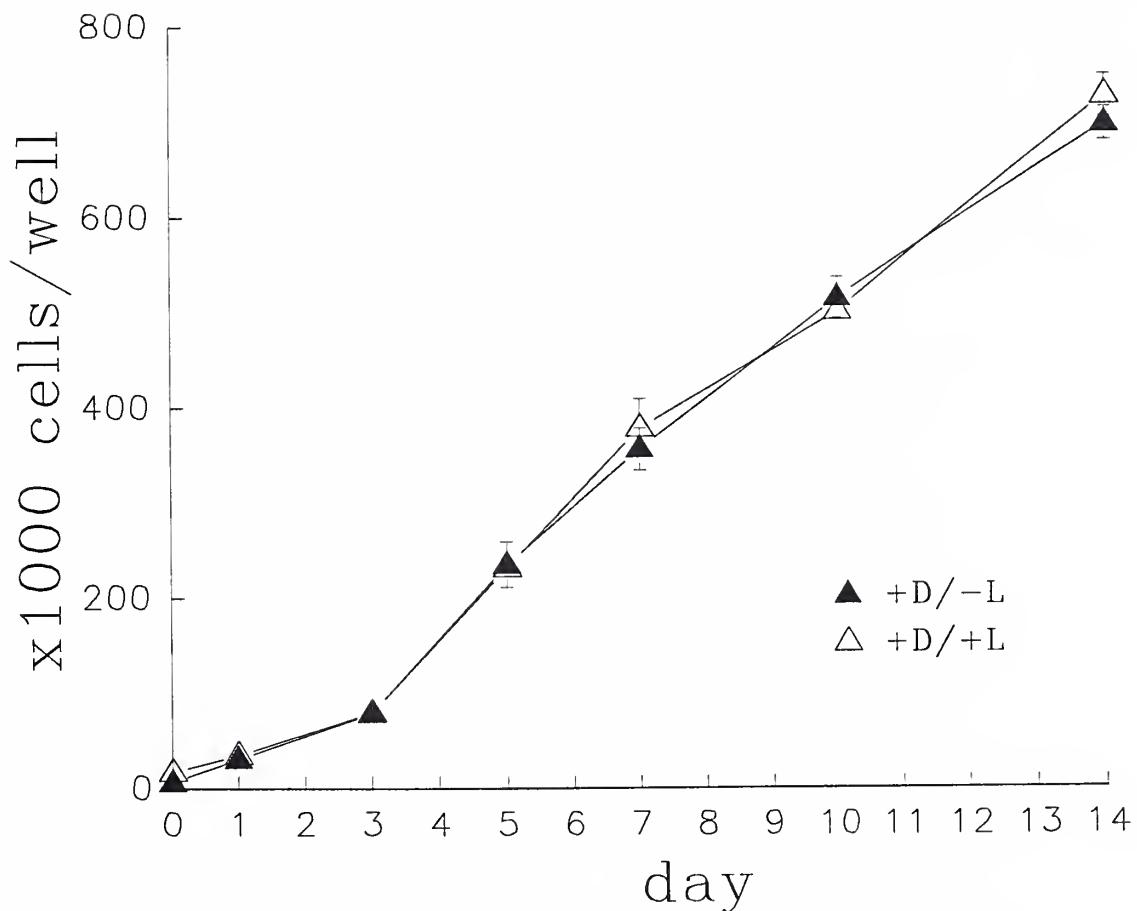
Figure 7-1. Proliferation curves for SMC treated at 1 µg/mL 8-MOP both with and without light. D = drug; L = light; hence, control cells are represented by dark triangles and treated cells by clear triangles. No difference is seen between control and treated cells.
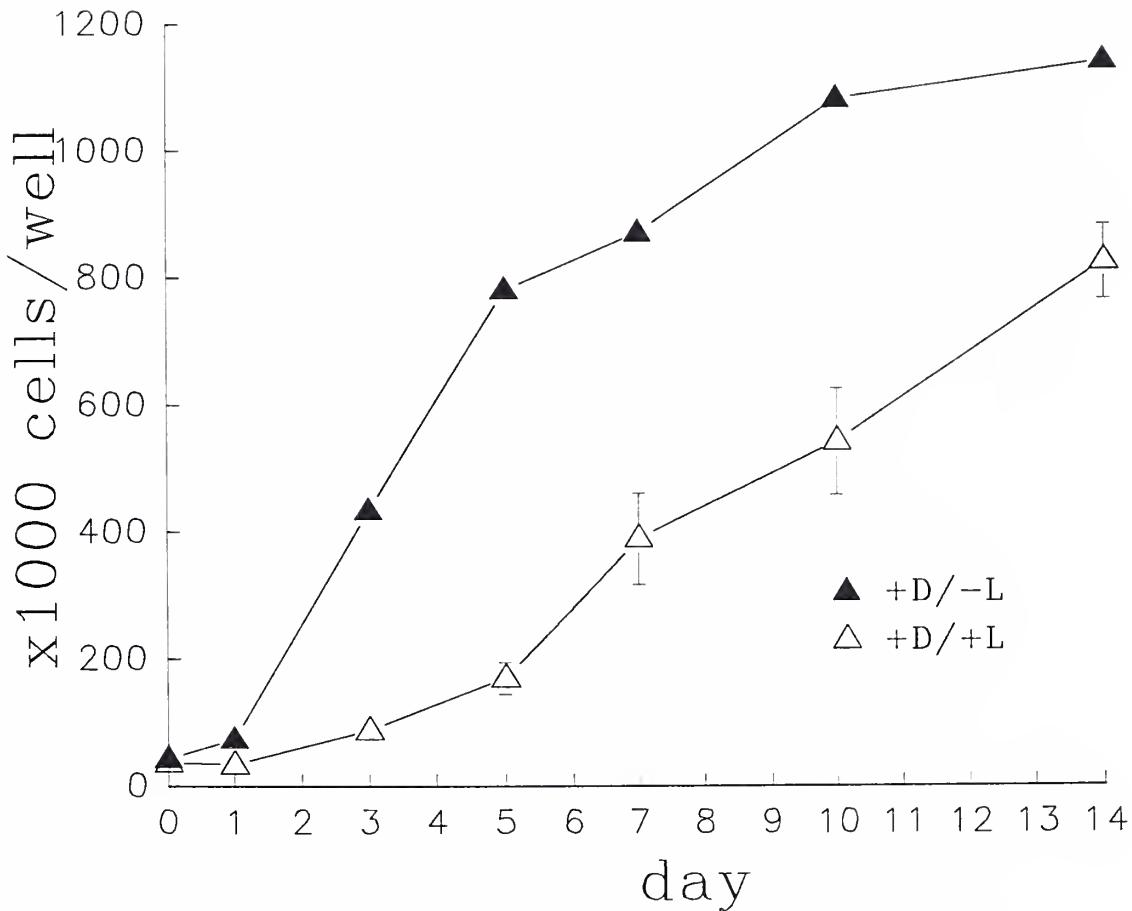
Figure 7-2. Proliferation curves for SMC treated at 20 µg/mL 8-MOP both with and without light. D = drug; L = light; hence, control cells are represented by dark triangles and treated cells by clear triangles. Transient and reversible inhibition of SMC proliferation is evidenced by the recovery in the growth curve for treated cells.

SMC; 50 μg/mL 8−MOP
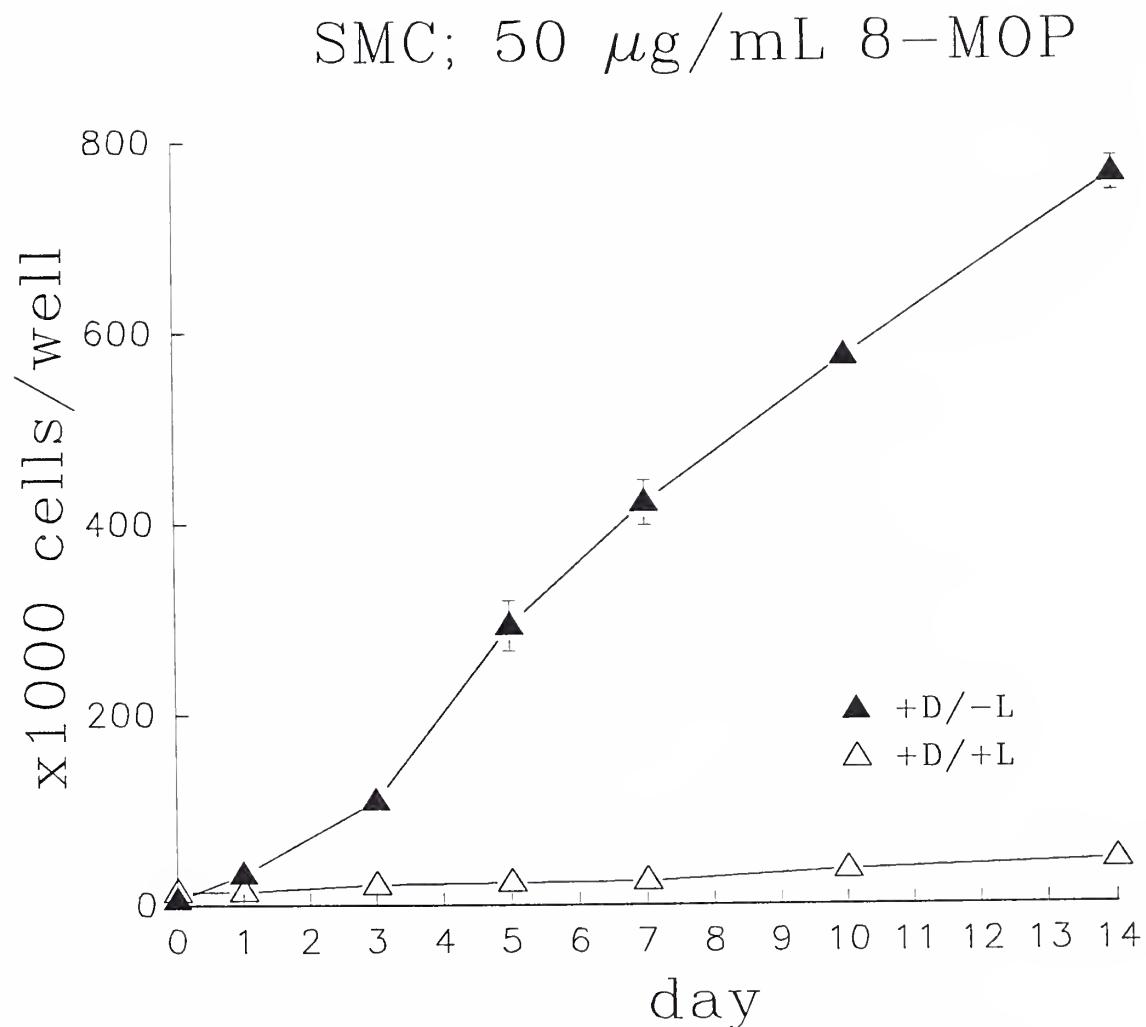
Figure 7-3. Proliferation curves for SMC treated at 50 μg/mL 8-MOP both with and without light. D = drug; L = light; hence, control cells are represented by dark triangles and treated cells by clear triangles. Treatment at this dose of 8-MOP appears to be cytotoxic to the SMC as no recovery is seen in the growth curve for treated cells.
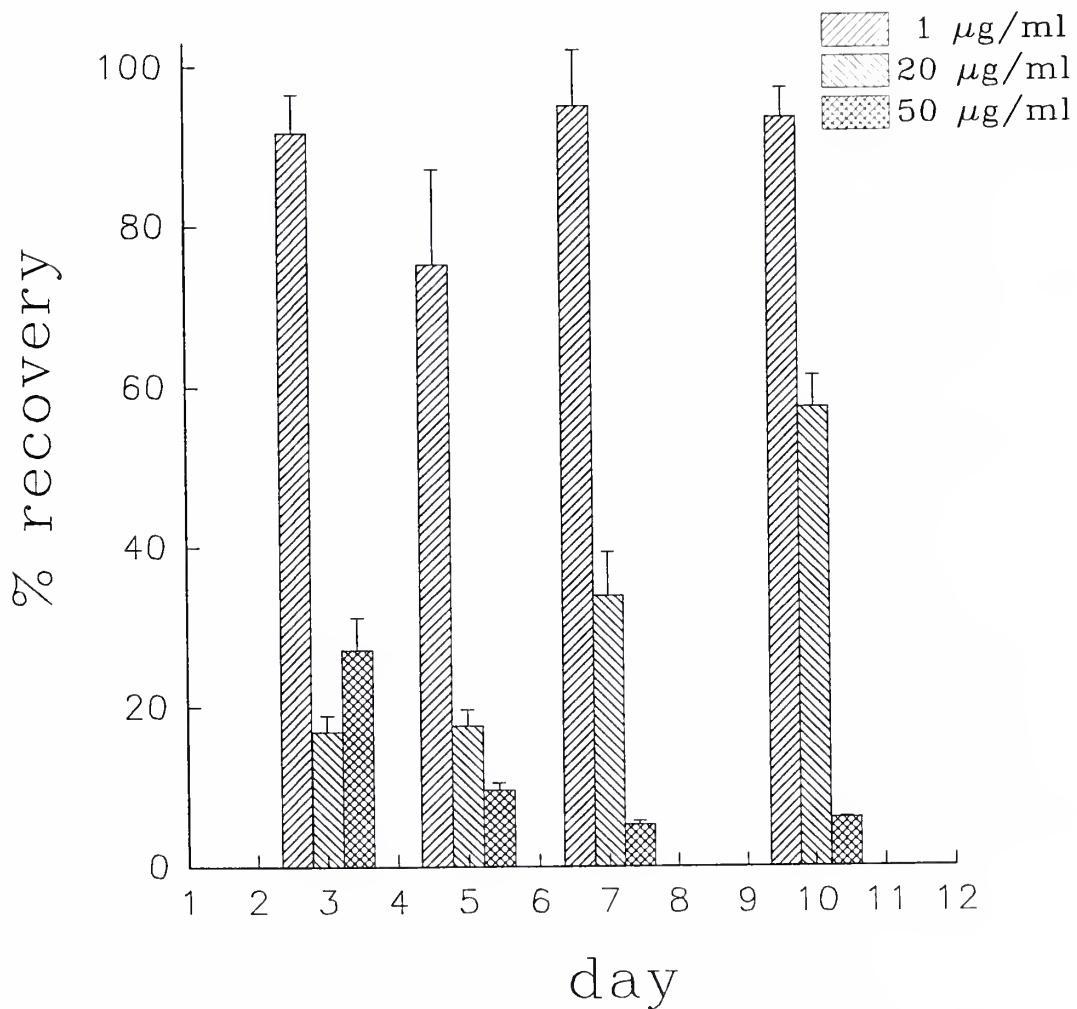
Figure 7-4. Summary graph of triplicate experiments performed on SMC at 1, 20, and 50 µg/mL 8-MOP. Percent recovery = number of treated cells/number of control cells. No effect is apparent at 1 µg/mL 8-MOP. Inhibition with gradual recovery of SMC proliferation is seen at 20 µg/mL 8-MOP. Cell death is seen at 50 µg/mL 8-MOP.

EC Proliferation

The effects of 12.0 J/cm^2 447 nm visible light-activated 8-MOP on EC proliferation are

shown in the following representative growth curves (Figures 7-5, 7-6, and 7-7). As seen

in the SMC experiments, the lowest dose of 8-MOP (1 μg/mL) had no effect on EC

proliferation (Figure 7-5). Likewise, the growth of EC treated with drug or light alone at

each dose was not significantly different from control, untreated EC (data not shown). At

both the intermediate and highest doses of 8-MOP (20 and 50 μg/mL, respectively),

however, inhibition of EC proliferation was transient and reversible, as evidenced by the

recovery in the growth curves (Figures 7-6 and 7-7). The results of triplicate experiments

per drug dose were combined and expressed as percent recovery for days 3, 5, 7, and 10

(Figure 7-8), where percent recovery represents the number of cells per aliquot divided by

the number of cells in the corresponding control aliquot. Differences in percent recovery

between cells treated with 1 μg/mL and the two higher doses were statistically significant

($p < 0.05$) only for day 5.
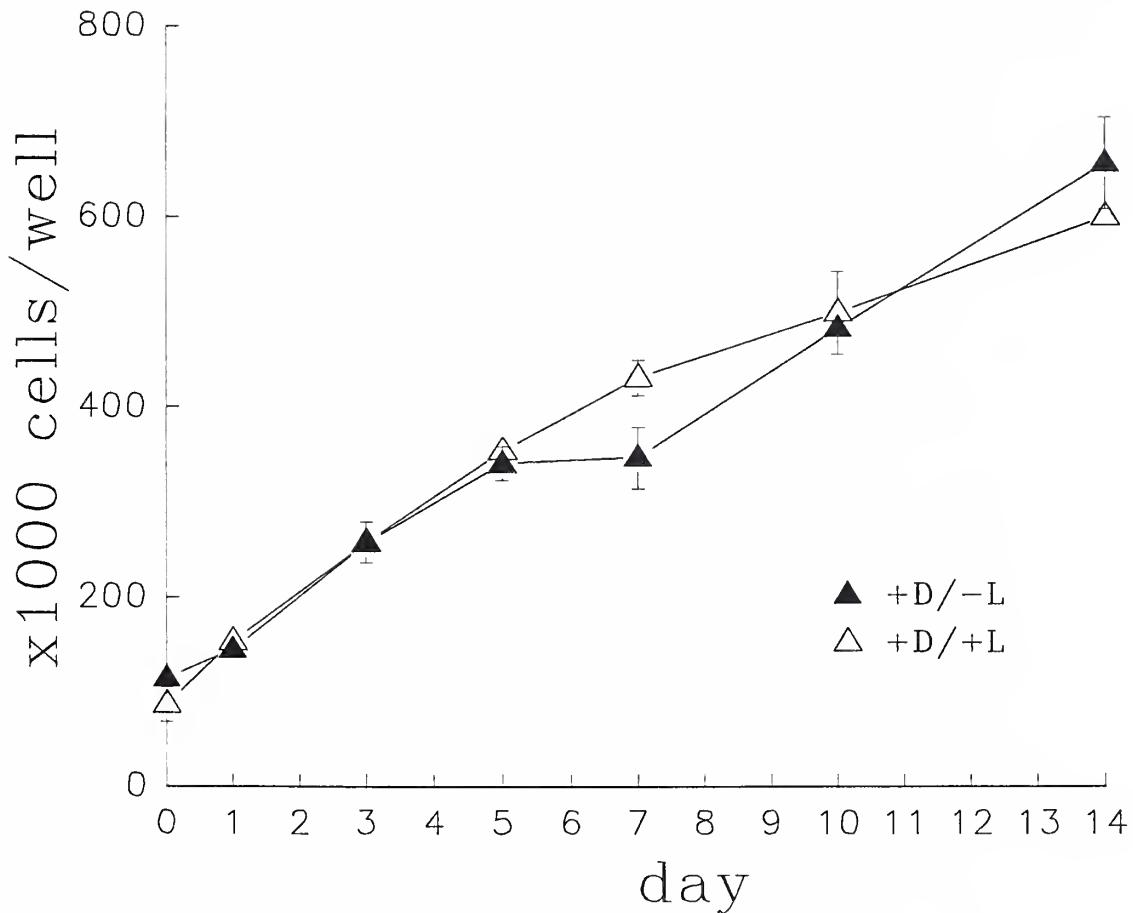
Figure 7-5. Proliferation curves for EC treated at 1 μg/mL 8-MOP both with and without light. D = drug; L = light; hence, control cells are represented by dark triangles and treated cells by clear triangles. No difference is seen between control and treated cells.

EC; 20 μg/mL 8−MOP
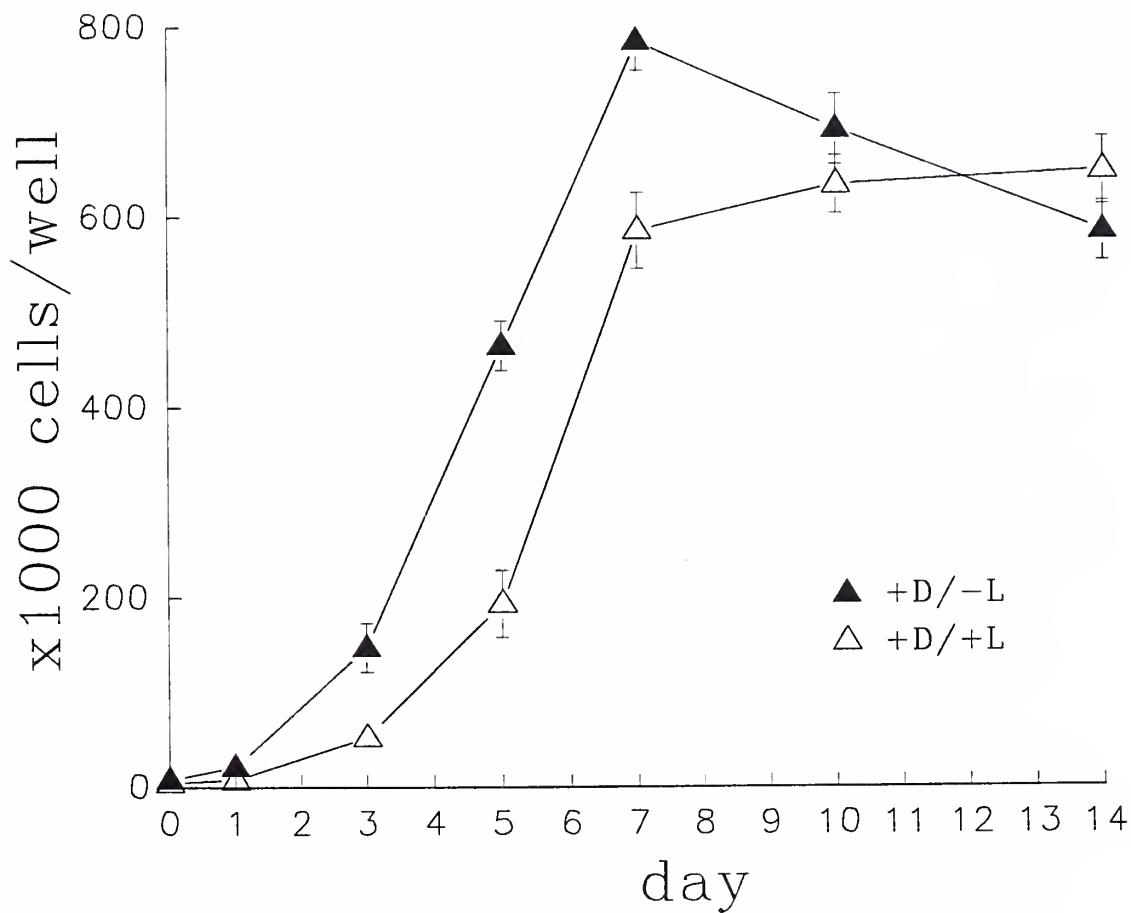
Figure 7-6. Proliferation curves for EC treated at 20 μg/mL 8-MOP both with and without light. D = drug; L = light; hence, control cells are represented by dark triangles and treated cells by clear triangles. Transient and rapidly reversible inhibition of EC proliferation is evidenced by the recovery in the growth curve for treated cells.

EC; 50 μg/mL 8−MOP
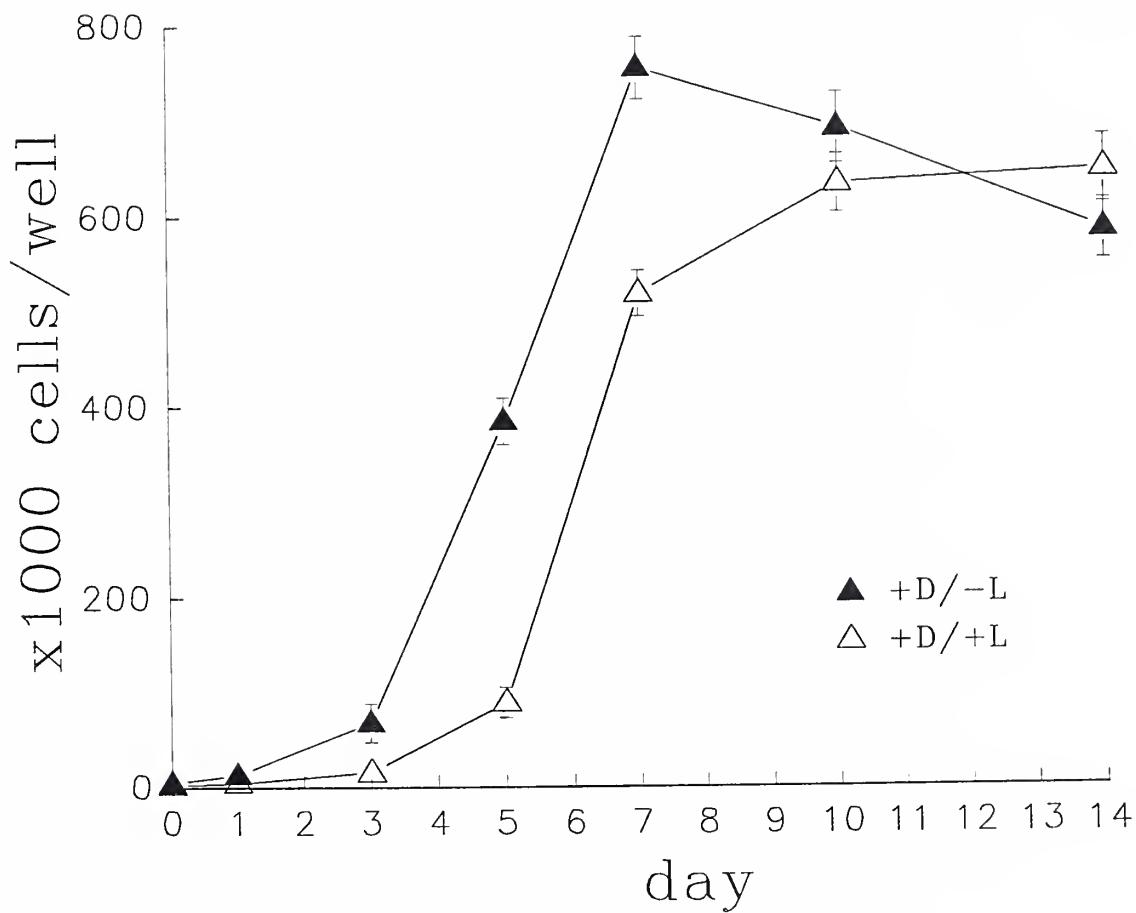

Figure 7-7. Proliferation curves for EC treated at 50 µg/mL 8-MOP both with and without light. D = drug; L = light; hence, control cells are represented by dark triangles and treated cells by clear triangles. Transient and rapidly reversible inhibition of EC proliferation is evidenced by the recovery in the growth curve for treated cells.
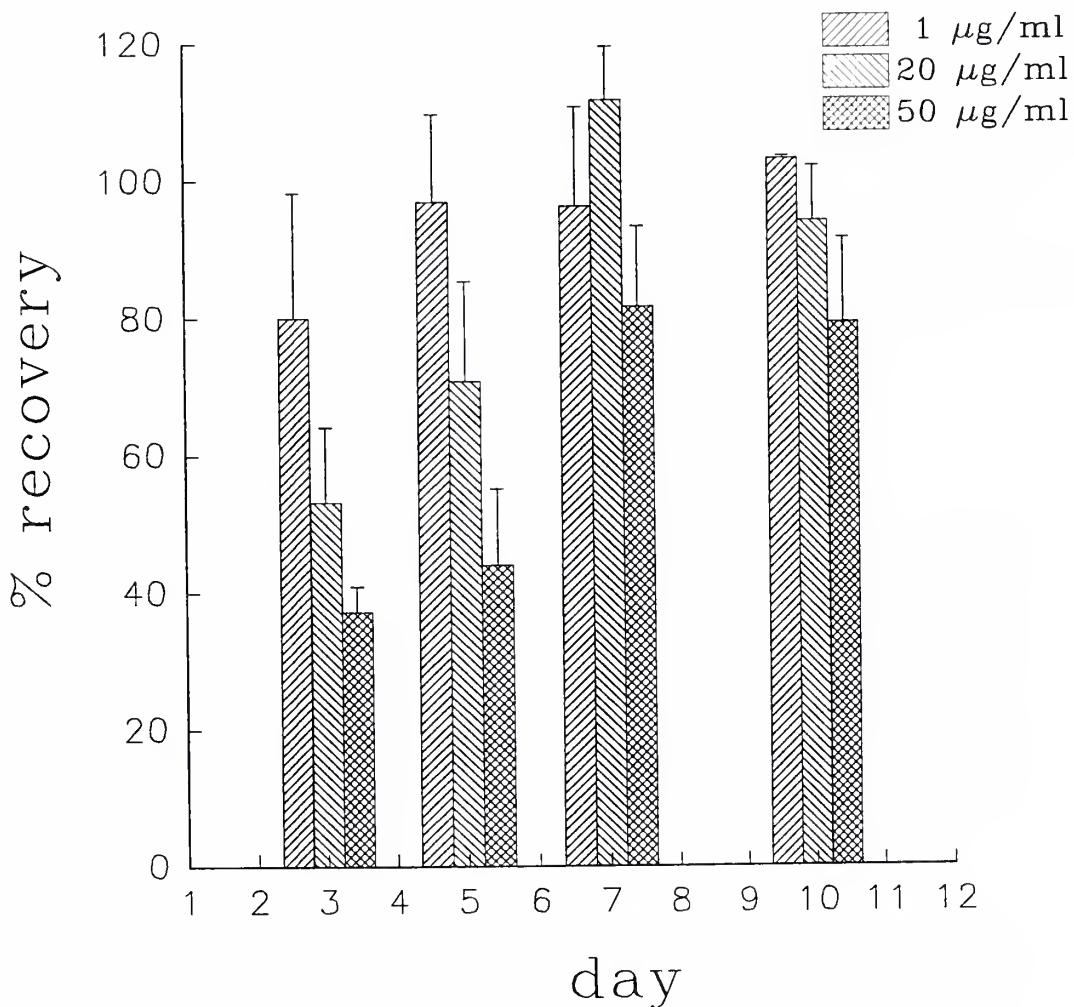
Figure 7-8. Summary graph of triplicate experiments performed on EC at 1, 20, and 50 µg/mL 8-MOP. Percent recovery = number of treated cells/number of control cells. No effect is apparent at 1 µg/mL 8-MOP. Inhibition with rapid recovery of EC proliferation is seen at both 20 and 50 µg/mL 8-MOP.

Cell Migration

Figure 7-9 demonstrates SMC migration on day 3 following treatment with 0, 1, 20, and

50 µg/mL 8-MOP. The photographs reveal that no concentration of 8-MOP was capable

of producing an inhibitory effect on SMC migration. Studies on EC also demonstrated a

lack of effect on cell migration, even at highest dose (data not shown). The extent of

migration observed in cells treated with mitomycin did not differ significantly from that

of cells not treated with mitomycin.

SMC Migration

Figure 7-9. Extent of SMC migration seen on day 3 after treatment with 0, 1, 20, and 50 µg/mL 8-MOP and 12 J/cm^2 447 nm light. No difference is apparent among these cells treated at different 8-MOP concentrations.

Cell Morphology

Figures 7-10, 7-11, and 7-12 represent a series of photographs which depict the morphology of treated and control SMC at days 5, 7, and 10. The photographs reveal cell densities consistent with those expected based on the SMC proliferation data. The majority of cells in all samples appeared healthy. While no difference in morphology was appreciated between cells treated with 1 μg/mL 8-MOP and control cells (Figure 7-10), morphologic examination of cells treated at 20 and 50 μg/mL 8-MOP revealed cells which were larger and phenotypically different from their respective controls (Figures 7-11 and 7-12). Additionally, this difference in morphology appeared to resolve over time in cells treated with 20 μg/mL 8-MOP (Figure 7-11) but not in those treated with 50 μg/mL 8-MOP (Figure 7-12).
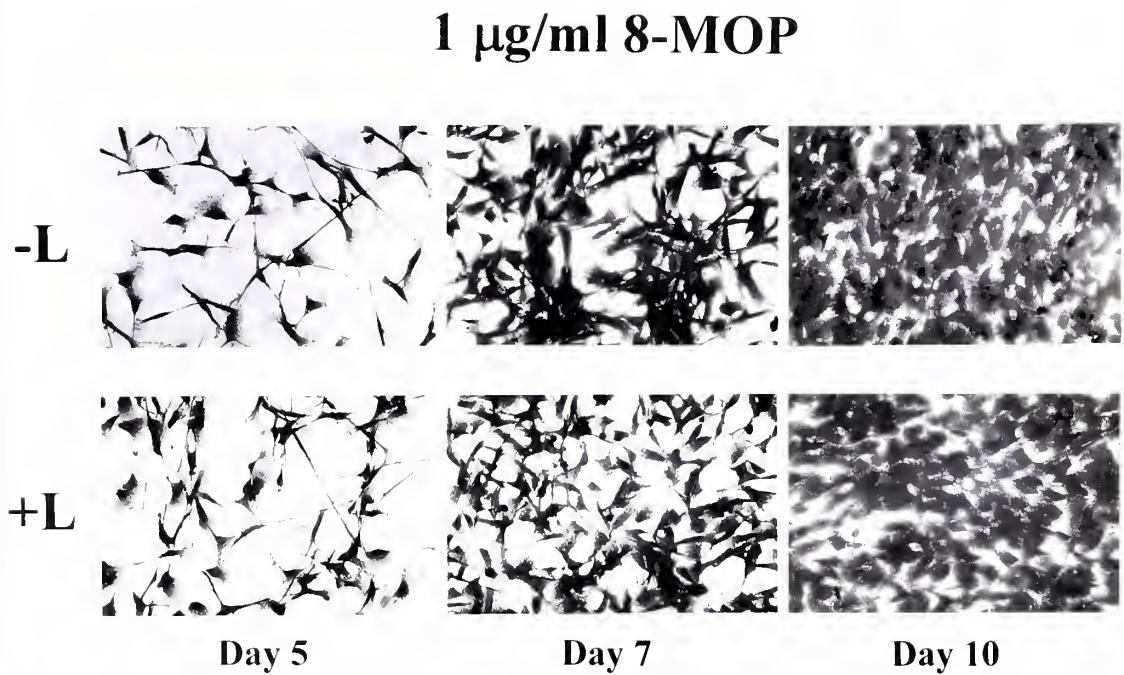
1 µg/ml 8-MOP

Figure 7-10. SMC morphology on days 5, 7, and 10 following treatment with 1 µg/mL 8-MOP. Cell densities are consistent with those expected based on SMC proliferation data. No difference in morphology is appreciable between control and treated cells.
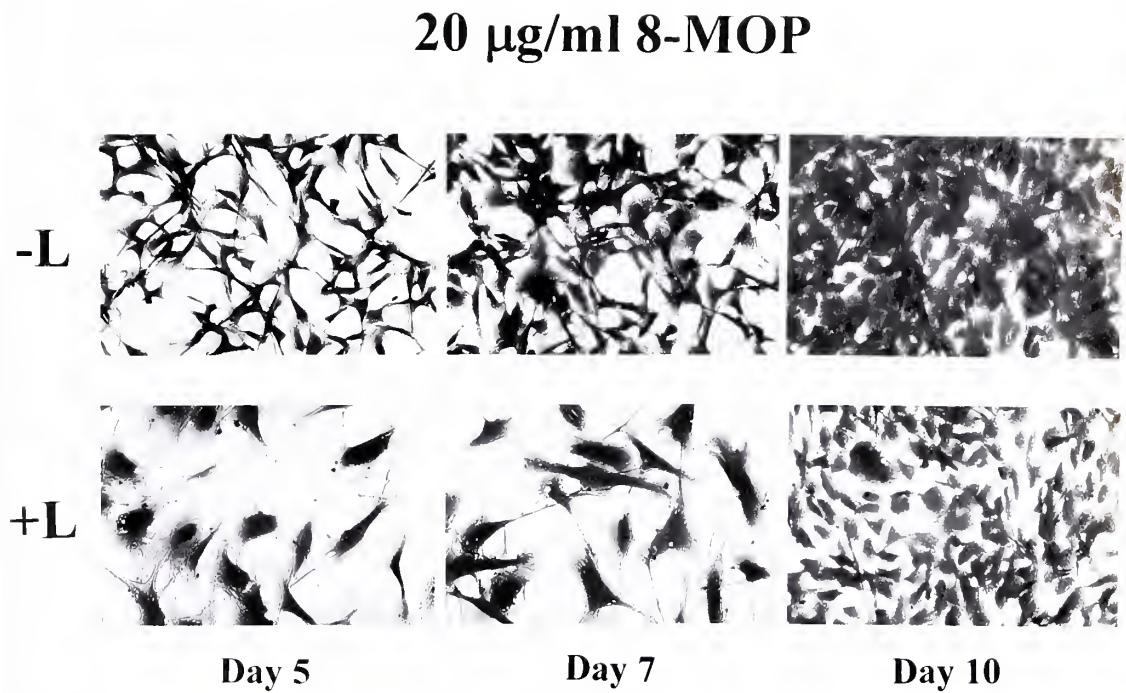
20 µg/ml 8-MOP

Figure 7-11. SMC morphology on days 5, 7, and 10 following treatment with 20 µg/mL 8-MOP. Cell densities are consistent with those expected based on SMC proliferation data. Treated cells appear larger and phenotypically different from control cells on day 5. This difference in morphology appears to begin to resolve by day 10.
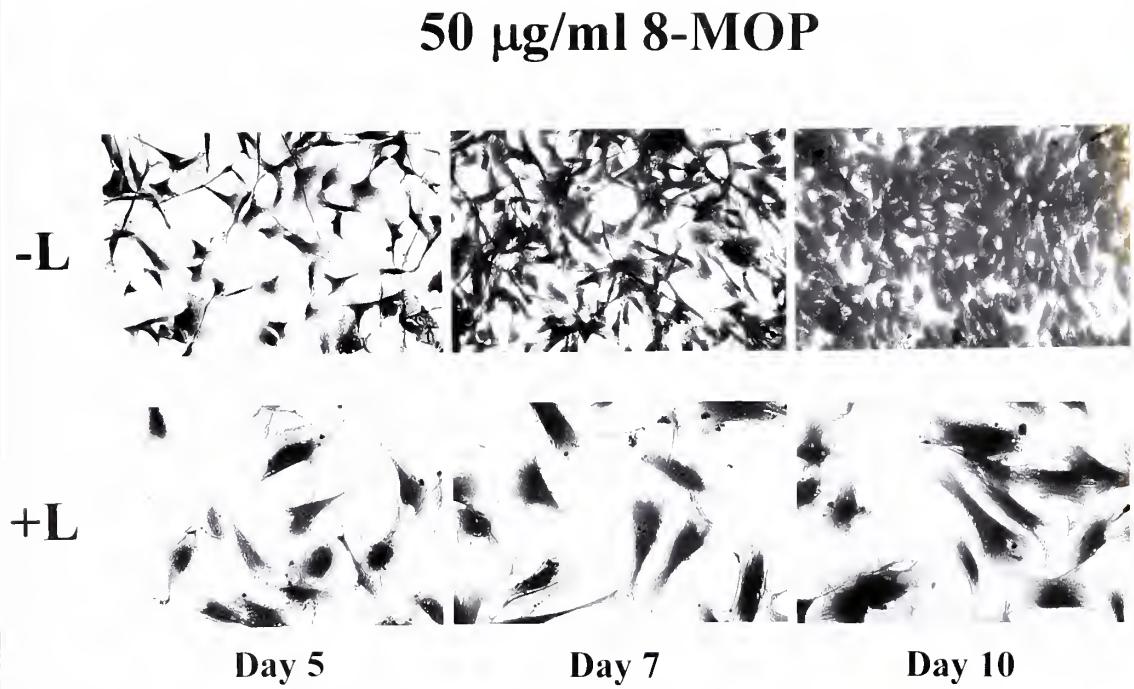
50 µg/ml 8-MOP

Figure 7-12. SMC morphology on days 5, 7, and 10 following treatment with 50 µg/mL 8-MOP. Cell densities are consistent with those expected based on SMC proliferation data. Treated cells appear larger and phenotypically different from control cells on day 5. This difference in morphology appears to persist.

Cell Size

Figure 7-13 shows a series of size distribution diagrams. The x-axis corresponds to the size of individual cells (measured in femtoliters, fL) while the y-axis represents the number of cells in each sample measured at that particular size. For each day, the size distribution for untreated control cells (-D/-L), 8-MOP only-treated control cells (+D/-L), visible light only-treated control cells (-D/+L), and cells treated with both 8-MOP and visible light (+D/+L) are shown. At any given day, the vertical dotted line represents the average (mode) size of the three different groups of control cells.

On day 3, the average size of untreated SMC was 1.70 fL, while the average size of untreated EC was 1.32 fL. For both SMC and EC, treatment with either 8-MOP or visible light alone failed to induce any significant change in cell size. Treatment with both 8-MOP and visible light, on the other hand, resulted in an increase in average cell size. This increase in cell size was maintained in SMC over the time course studied, with only a slight decrease in cell size detected by day 14. The increase in EC size, however, rapidly resolved with a return to control sizes by day 10.

These changes in cell size were plotted over a time course (Figure 7-14). For SMC, average cell size increased to 2.50 fL by day 5 and then plateaued at 3.20 fL by day 10. A different pattern was observed in EC, which increased in average size to 3.80 fL by day 5 but returned to near control size (1.30 fL) by day 10.

Figure 7-13. Cell size distribution diagrams. x-axis = size of cells, measured in femtoliters (fL); y-axis = number of cells measured at the given size. D = drug; L = light. The four rows represent size distribution diagrams for untreated control cells (-D/-L), 8-MOP only-treated control cells (+D/-L), visible light only-treated control cells (-D/+L), and 8-MOP and visible light-treated cells (+D/+L). The vertical dotted line represents the average (mode) size of the three different groups of control cells.

EC

-D/-L
+D/-L
-D/+L
+D/+L

DAY 3
DAY 7
10
DAY

SMC

-D/-L
+D/-L
-D/+L
+D/+L

DAY 3
DAY 7
10
DAY
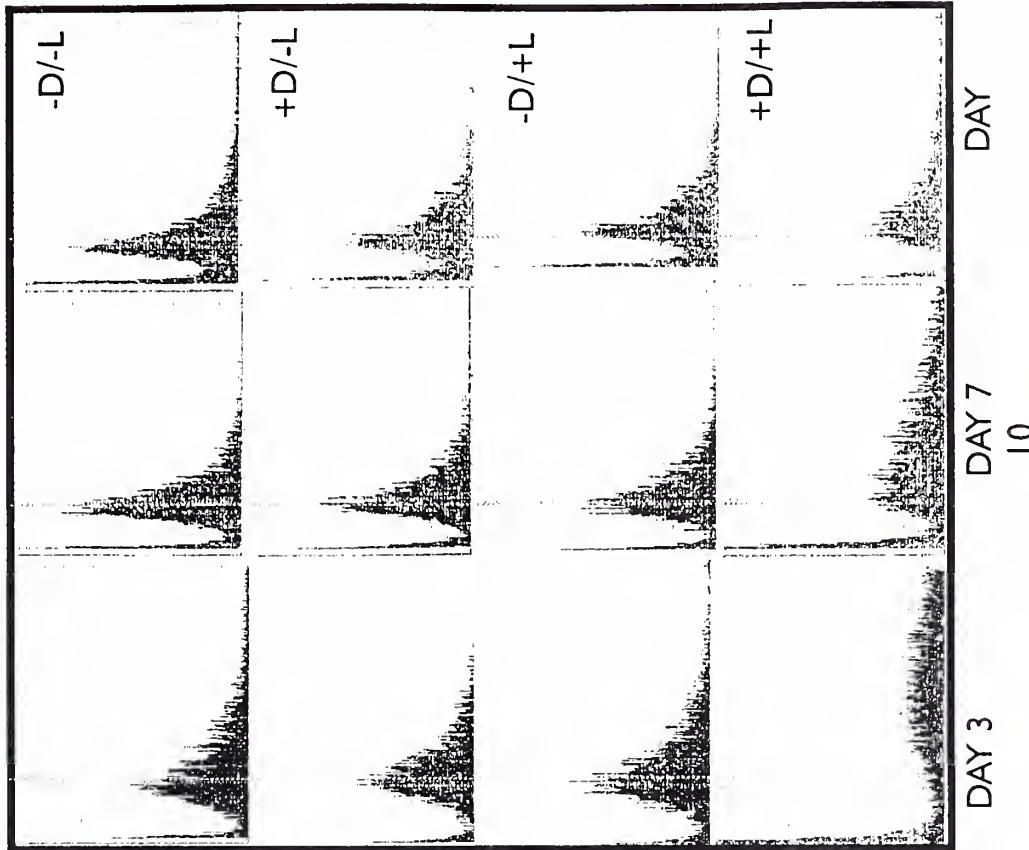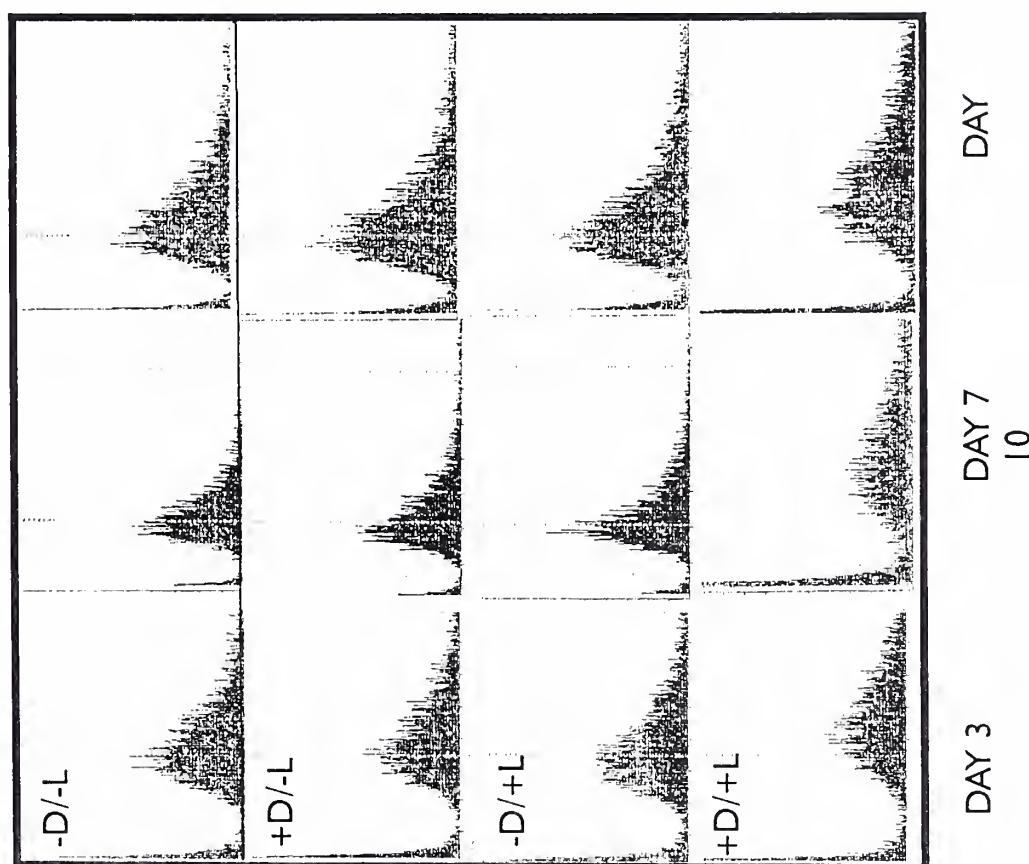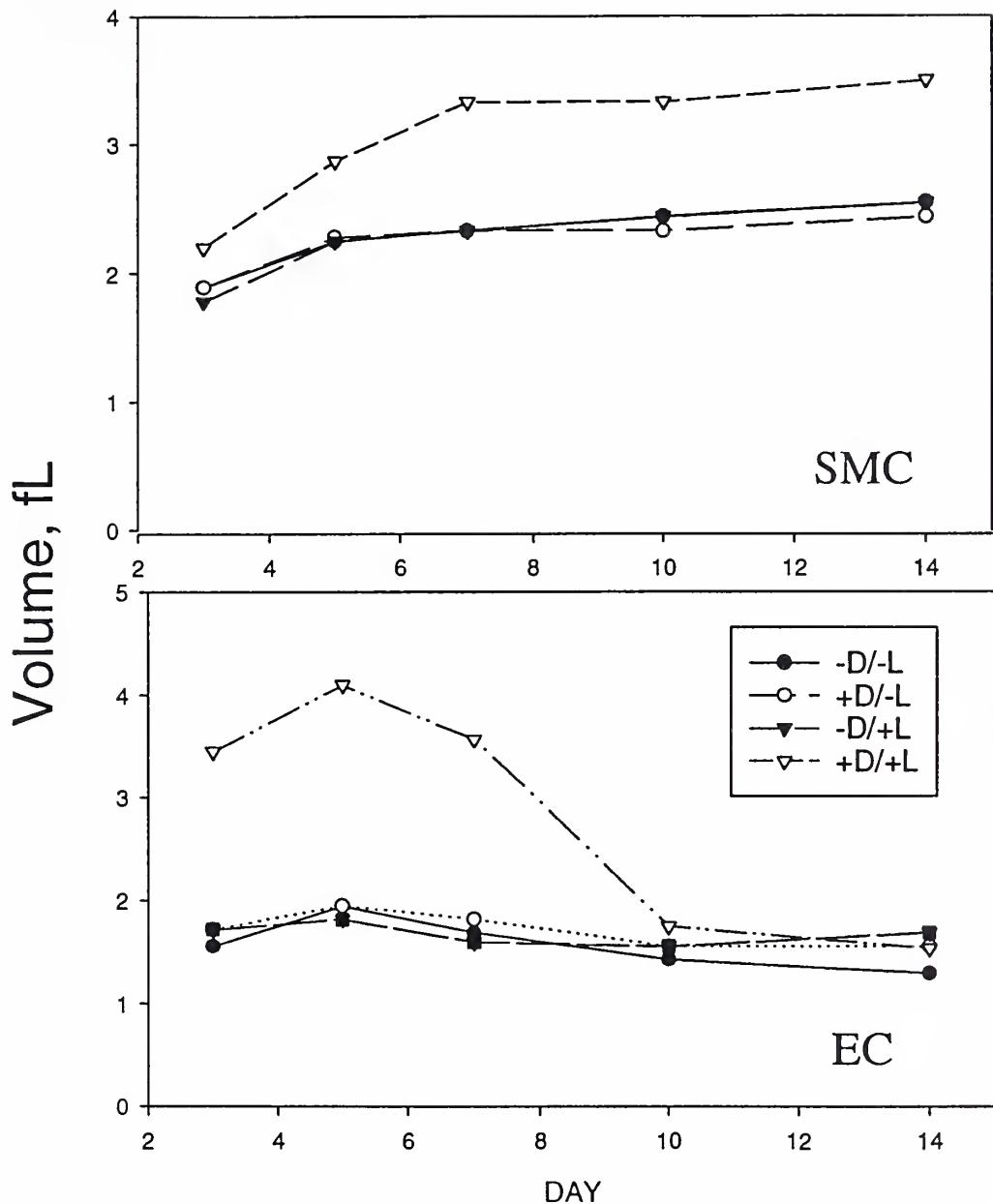
Figure 7-14. Changes in cell size (volume) followed over the course of 14 days. Average cell size appears to increase and plateau in SMC treated with both drug and light (clear triangles). In EC treated with both drug and light, average cell size initially increases but rapidly returns to control sizes by day 10 (clear triangles).

Photoadduct Formation

SMC and EC treated with 20 µg/mL 8-MOP and 32 J/cm^2 447 nm light revealed

comparable levels of photoadduct formation. 110 adducts per million base pairs were

found in SMC, while 200 adducts per million base pairs were seen in EC. Relative

formation of the different types of photoadducts (4', 5'-monoadducts; 3, 4-monoadducts;

and crosslinks) were similar between the two cell types as demonstrated in the following

high-performance liquid chromatography (HPLC) curves (Figure 7-15).

CPM

Fraction Number
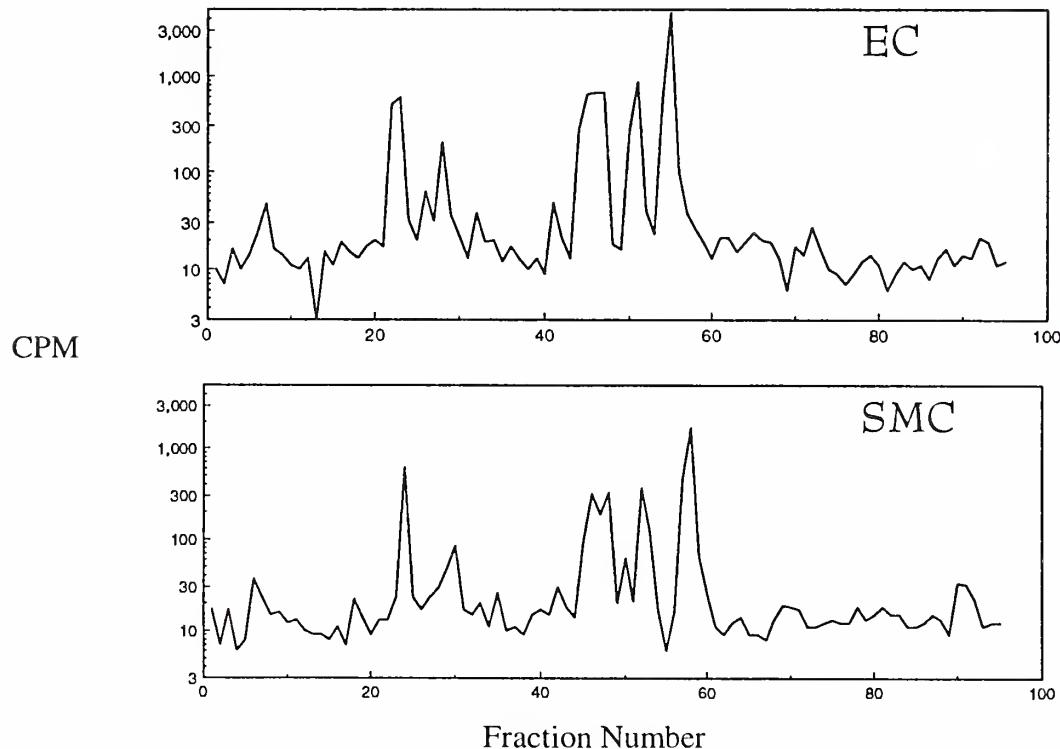
Figure 7-15. Graphs comparing photoadduct formation in EC and SMC. CPM = counts per minute. Late-eluting 8-MOP-thymdine photoadducts were detected by liquid scintillation analysis. Photoadduct distributions between the two cell types were similar.

8.   Discussion

Discussion of Results

Cell Proliferation. The present study demonstrates that 8-MOP photoactivated with 447

nm visible light can reversibly inhibit both SMC and EC proliferation *in vitro* in a dose-

dependent fashion. The study also reveals that different 8-MOP dosages cause a range of

effects in SMC proliferation, from no effect to reversible cytostasis to cytotoxicity. These

selected dosages appear to have significantly attenuated effects on EC compared to SMC.

The rapidly reversible inhibition of EC proliferation observed in the experiments, even at

high 8-MOP concentrations, bears clinical significance in light of previous studies which

have demonstrated that a functionally intact endothelium contributes to the inhibition of

SMC proliferation (Ip et al., 1991). The fact that photochemotherapy with 8-MOP and

visible light can selectively inhibit SMC proliferation without causing damage to EC

immediately suggests this treatment regimen as a potential solution to the problem of post-

angioplasty restenosis.

Cell Migration. As intimal hyperplasia involves not only SMC proliferation but also

SMC migration, experiments evaluating the effects of 8-MOP photoactivated with 447

nm visible light on cell migration were performed. Unlike the effects observed in SMC

and EC proliferation, however, no inhibition of either SMC or EC migration was found.

Nevertheless, while both SMC migration and proliferation are involved in the neointima

formation which occurs after angioplasty, it is known that SMC proliferation contributes more materially to the process.

It has been estimated that only about one half of the SMC which migrate into the intima after angioplasty undergo further proliferation. The remaining SMC do not. Those SMC which do continue to proliferate undergo approximately three rounds of division, thereby constituting eight ninths of the final neointimal cell population (Clowes and Schwartz, 1985). This suggests that SMC proliferation has a significantly greater impact on restenosis than SMC migration; hence, the finding that phototherapy with 8-MOP and visible light fails to inhibit cell migration does not disqualify this treatment regimen as a potential solution to the problem of post-angioplasty restenosis.

Cell Morphology. Despite its lack of effect on cell migration, the combination of 8-MOP and visible light was found to affect SMC morphology. It is interesting to note that just as different effects in SMC proliferation were observed in response to different 8-MOP dosages, a range of effects in SMC morphology was also seen in response to different 8-MOP dosages. While no morphological differences were appreciated between SMC treated with 1 μg/mL 8-MOP and their controls, SMC treated with 20 μg/mL and 50 μg/mL 8-MOP appeared larger and phenotypically different from their respective controls. Moreover, this difference in morphology appeared to resolve over time in the cells treated with 20 μg/mL 8-MOP but not in those treated with 50 μg/mL. Whether this

range of effects observed in cell morphology is somehow related to the range of effects seen in SMC proliferation remains to be elucidated.

Cell Size. Additional studies employing a Channelyzer capable of measuring cell sizes (volumes) were conducted to corroborate the cell enlargement induced by treatments with 8-MOP and 447 nm visible light observed in the morphology experiments. Cell size measurements confirmed that treatment with 20 µg/mL 8-MOP causes an increase in average cell size for both SMC and EC. Unlike the findings noted in the morphology studies, however, the increase in SMC sizes did not resolve over time but instead persisted. The increase in EC sizes, on the other hand, appeared to resolve by day 10.

The reason for these differences in cell size changes over time noted between the morphology experiments and cell size experiments is unclear but may be due to the fact that morphology studies assess cell sizes on a two-dimensional level, while a Channelyzer measures cell sizes in three dimensions. The difference in ability of SMC and EC to return to control sizes following the same treatment represents an important finding and may be related to the differences observed in the cell proliferation experiments, as an attenuated response to treatment with the same 8-MOP dosage is again observed in EC compared to SMC.

Photoadduct formation. As anticipated, the number of photoadducts measured in SMC and EC were within the same order of magnitude. Given the comparatively attenuated effects of the treatments on EC proliferation and size, one might have expected fewer photoadducts to have been found in EC than SMC. Surprisingly, the number of

photoadducts measured in EC was nearly twice that measured in SMC. This suggests that the attenuated effects observed in EC are not the result of the formation of fewer photoadducts.

Potential Future Experiments

Comparison between SMC and EC proliferation curves reveals that photochemotherapy with high doses of 8-MOP is cytotoxic to SMC but yields only transient cytostasis in EC. The reason for these differences in response between SMC and EC to identical photochemotherapeutic treatments remains to be determined. As it had been presumed that inhibition of cellular proliferation could be directly related to extent photoadduct formation, the decreased sensitivity of EC to 8-MOP treatments was initially hypothesized to be the result of decreased photoadduct formation. The preliminary study on photoadduct formation, however, revealed that EC forms nearly twice the number of photoadducts as SMC.

As this observation is based on a single experiment, repeat experiments on photoadduct formation are in order. Repeat studies may confirm that photoadduct formation is indeed consistently greater in EC or may reveal that the difference in photoadduct formation between SMC and EC observed in the pilot study falls within normal variance.

Assuming that the greater photoadduct formation observed in EC is genuine, experiments designed to explicate the fact that EC are less sensitive to treatments with 8-MOP and visible light despite more extensive photoadduct formation should be undertaken. As differences in DNA repair rates which would allow EC to restore its proliferative

functionality more rapidly than SMC might account for this discrepancy, experiments comparing photoadduct formation levels in SMC and EC over a time course could be performed. These studies would help to characterize the DNA repair kinetics of the two cell types and might reveal differences. Specifically, they might demonstrate that EC possess more efficient DNA repair machinery than SMC.

However, should these experiments reveal that EC exhibit greater photoadduct formation than SMC at all time points, the hypothesis that inhibition of cell proliferation is directly related to extent photoadduct formation would have to be challenged. Further experimentation might reveal that extent photoadduct formation is only one of a number of factors which contribute to the inhibition of cell proliferation.

Another issue which could potentially be addressed in future experiments is the question of whether photochemotherapy with 8-MOP and visible light would yield similar results in human coronary cells. The difference in response seen between bovine aortic SMC and EC already suggests that dissimilar cell types may respond differently to the same treatment. Thus, studies performed on human coronary cells *in vitro* would be useful.

While photochemotherapy with 8-MOP and visible light has been shown to dose-dependently inhibit proliferation of SMC and EC *in vitro*, it would also be valuable to evaluate the effects of this treatment regimen on vascular cells *in vivo*. The experimental methods employed in the present study artificially isolate SMC from EC. Recall that in the normal coronary artery, these cells are separated by only a thin, fenestrated internal elastic membrane (see "The Normal Coronary Artery," Chapter 1). Thus, in the live

model, these cells interact with each other and affect one another's functional capacities. Hence, *in vivo* studies designed to corroborate the beneficial effects of phototherapy with 8-MOP suggested by our *in vitro* experiments would also prove useful.

Finally, studies examining the effects of visible light-activated 8-MOP on cell functions other than proliferation and migration might prove beneficial. For example, experiments designed to assess the effects of these treatment regimens on the ability of SMC and EC to synthesize proteins could be performed. If protein synthesis were inhibited, follow-up studies could be performed to assess recovery of synthetic function. These types of experiments on the effects of visible light-activated 8-MOP on vascular cell function would further elucidate the suitability of this treatment modality for clinical trials.

Significance of Results

The clinical significance of the present study is related to the established long-term safety of 8-MOP, the availability of 447 nm lasers, the ability to target therapy to the angioplasty site, and the potentially reversible nature of the effects of therapy.

As mentioned previously, 8-MOP exhibits a rapid clearance which results in minimal clinical phototoxicity. Its common use in the treatment of dermatologic diseases has established its long-term safety. Moreover, the significant reduction in extent of crosslink formation seen at photoactivation with 447 nm light may render photochemotherapy with 8-MOP at this wavelength a greater therapeutic index.

Perhaps this reduction in crosslink formation contributes to the rapidity of the proliferative functional recovery observed in SMC treated with intermediate dose (20 µg/mL) 8-MOP. The cytostatic effect of this treatment regimen without cytotoxicity represents a theoretical advantage over cytotoxic therapies for the prevention of restenosis. SMC necrosis caused by cytotoxic therapies could compromise the mechanical integrity of the arterial wall and result in the formation of an aneurysm. Moreover, cytokines released by damaged or dying SMC could stimulate a secondary local inflammatory response and cause an exacerbation of the cellular proliferative response (Sumpio et al., 1994).

As the migration and proliferation of SMC stimulated by angioplasty begins as early as 24 to 48 hours after the procedure and lasts for approximately one week (Forrester et al., 1991; Ip et al., 1991), a transient, reversible inhibition of SMC proliferation may represent an optimal solution to the problem of post-angioplasty restenosis. The present study demonstrates that 8-MOP photoactivated with visible light is capable of inhibiting SMC proliferation, presumably via photoadduct formation. Excision of these photoadducts by DNA repair mechanisms would allow for the eventual return of proliferative capacity and could result in the restoration of SMC functionality at a time when the stimulus to proliferate has waned.

Currently available technology permits photochemotherapy with 8-MOP and visible light to be directed to a discrete site on the vessel wall by means of local drug and/or light delivery. While 8-MOP has traditionally been administered systemically (both orally and

intravenously) for other therapeutic applications, high local concentrations of the drug

could be attained via intra-arterial administration with a double-balloon catheter.
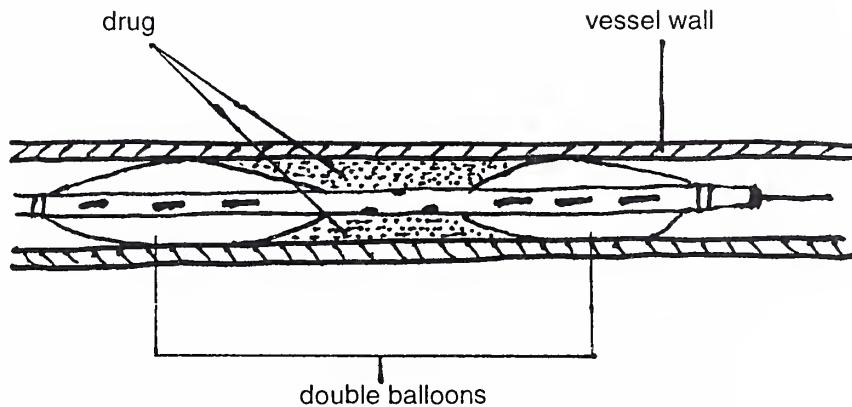
Figure 8-1. Schematic of a double-balloon catheter demonstrating how high local concentrations of drug could be achieved with this device.

Intra-arterial light irradiation of a segment of the vessel wall could then be achieved using

a recently described fiber-optic balloon catheter, which has been clinically evaluated for

simultaneous balloon dilatation and arterial wall irradiation (Spears et al., 1990).

9. Conclusion

In conclusion, SMC proliferation constitutes a major mechanism by which restenosis after angioplasty occurs. A therapeutic regimen capable of inhibiting SMC proliferation both selectively and reversibly might represent an ideal solution to the problem of post-angioplasty restenosis. As the present study demonstrates that photochemotherapy with 8-MOP and visible light possesses these characteristics, this treatment regimen may represent a novel approach to the prevention of post-angioplasty restenosis.

10. References

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